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## SOME EFFECTS OF CORPUS LUTEUM EXTRACTS AND OF SYNTHETIC PROGESTERONE ON YOUNG MALE ALBINO RATS[1,2]

(One plate)

JULE KITCHELL LAMAR
Hull Zoölogical Laboratory, University of Chicago

THE corpus luteum is an endocrine gland which, by means of its internal secretion, affects many processes in the postpuberal female physiology of mammals (Allen, Hisaw, and Gardner, 1939). These processes may be similarly affected in the absence of all luteal tissue by administration of crude potent luteal-tissue extracts or of pure crystalline progesterone, the so-called "hormone of the corpus luteum" (Allen and Corner, 1930; Corner and Allen, 1936). Whereas, all other sex hormones, both male and female, have been shown to have more or less bisexual properties, the corpus luteum hormone has long been considered specifically female in its action (Korenchevsky, 1937). With the advent of crude estrogen-free corpus luteum extracts (progestin, Allen and Meyer, 1933), pure crystalline hormone from extracts (Slotta, Ruschig, and Fels, 1934; Allen and Wintersteiner, 1934; Butenandt, Westphal, and Hohlweg, 1934), and synthetic progesterone (Fernholz, 1934; Butenandt and Schmidt, 1934; Butenandt and Westphal, 1934) it became more and more feasible to put to the test this assumption of sex specificity of the corpus luteum hormone. The experiments reported herewith were designed to determine whether definite effects follow the administration of progestin and progesterone in male rats.[3]

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[2] Presented before the American Society of Zoölogists at the Indianapolis meeting, December, 1937. Abstract in *Anatomical Record*, 70, Supp. 1, pp. 45–46.

[3] It is a genuine pleasure to express my grateful appreciation to Professor Carl R. Moore for suggesting this problem and for his ever helpful co-operation and stimulating advice. To Professor W. C. Allee, my best thanks are due for use of essential equipment for hormone extractions, and to Professor Sewall Wright, my sincere gratitude for aid in the statistical analysis of the results. Grateful acknowledgment is hereby offered to the following for generous supplies of hormone preparations: Dr. Gregory Stragnell, of Schering Corporation, for Proluton and Oreton; Dr. J. F. Cartland, the Upjohn Company, for Progestin; and Dr. Oliver Kamm, Parke, Davis and Company, for Theelin.

## Materials and Methods

We have used in these experiments: (1) sow corpus luteum extracts prepared in this laboratory ("Progestin I" and "Progestin II"); (2) sow corpus luteum extracts from the Upjohn Company ("Progestin III"); and (3) synthetic progesterone (Proluton, Schering). Progestin preparations had been subjected to treatment (Allen and Meyer, 1933) for removal of estrogens. Estrone (Theelin, Parke, Davis and Company) and testosterone (Oreton, Schering) were used for a comparison of their effects with those of corpus luteum hormone.

All corpus luteum hormone preparations were assayed on adult virgin female rabbits according to the techniques of Corner and Allen (1929) and Allen (1930).

Albino rats used in these experiments were of two kinds, namely, (1) an inbred strain derived from the regular colony, and (2) rats from the regular colony, used when inbred rats were not available. Female mice used as recipients of pituitary implants were from the small colony maintained in this laboratory for such purposes. Rabbits used for the assay of corpus luteum hormone were young mature virgin does purchased from a dealer and kept isolated in separate cages for a period of 1 month or more before treatment.

One hundred and ninety rats, 47 mice, and 21 rabbits were used in this investigation.

Methods standard in this laboratory for operation, injection, implantation of pituitaries, and autopsy were adopted. Histological methods were simple, involving paraffin imbedding and simple staining.

With the exception of assay work on rabbits, the principle of littermate controls has been used throughout. Analyses of organ weight data were carried out using Student's method.

## Experimental Results

### Progestin on normal young male rats

To determine the possible effects of corpus luteum hormone on normal rats a wide dose range of progestin was tried on four litters of various ages (see Table 1). Young

TABLE 1

| Expt. No. | Dose Range (Rb. U. [Corner], Daily) | Progestin Sample Used | Injection Period (Days) | | Age at Autopsy (Days) |
|---|---|---|---|---|---|
| | | | Age | Total | |
| I | 1, 1/5, 1/20 | I | 38–51 | 14 | 52 |
| II | 1, 1/2 | I | 25–39 | 15 | 40 |
| III | 1, 1/2 | I | 17–31 | 15 | 32 |
| IV | 3 | II | 25–39 | 15 | 40 |

rats were used, for it is well known that young animals are more responsive to sex hormone treatments than are adults. In the first experiment a dose range of 0.05–1.0 rabbit unit (Rb. U.) (Corner) was tried, with the idea that some notion of threshold, and perhaps a quantitative difference in response, might be obtained should any effects of progestin be found. In the last experiment the largest dose which our limited supply would permit was given to one animal.

### WEIGHT EFFECTS

Results of these experiments were, in the main, negative. Organ weights of treated animals differed from those of oil-injected littermate controls in but few cases. Even where some organ weight difference was observed, it could be considered only as suggestive because of the small number of animals used (one injectate at each dose-level). A brief outline of weight effects is detailed in Table 2.

TABLE 2

EFFECTS OF PROGESTIN ON ORGAN WEIGHTS ON THE BASIS OF ABSOLUTE WEIGHT AND PERCENTAGE OF BODY WEIGHT

| Expt. No. | Percentage of Decrease (−) or Increase (+) in Organ Weights of Treated versus Control Animals | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Body Weight | Testis | Preputial Glands | Ventral Prostate | Seminal Vesicles | Coagulating Glands | Periurethral Tissue | Cowper's Glands | Adrenal Glands | Thymus Gland | Thyroid Gland | Pituitary Gland |
| I..... | o* | o | —† | o | o | o | — | o | o | — | o | o |
| II..... | o | o | — | o | o | o | — | o | o | o | o | o |
| III..... | o | −42, −46 | — | +51, +61 | +60, +70 | +59, +69 | — | o | o | −19, −24 | o | o |
| IV..... | o | o | +50, +80 | +99, +135 | o | o | o | o | o | o | o | o |

* Zero (o) indicates essentially no difference.
† Dash (—) indicates no weight taken.

A study of these data shows that in only one experiment (III), run from day 17 to day 32, was there any effect of injections on testis weight. This effect was a slowing of the growth-rate of the testis. At the same time there seemed to be a slowing of the growth-rate of the thymus and some stimulating effect on the growth of ventral prostate, seminal vesicles, and coagulating glands. In one other case (Expt. IV) the ventral prostate was distinctly heavier than that of the control. Preputial glands were heavier also. These weight differences were confined to organs which respond to androgens by similar changes. Therefore, they suggested a weak androgen-like effect of the injected material. None of the weight effects noted was very pronounced, and not enough animals were used to make the study of any response statistically significant.

### EFFECTS ON SPERM MOTILITY

Sperm smears were taken at the time of autopsy of animals in Experiment I. In the animal which had received the highest dosage of progestin, spermatozoa were more numerous and more motile than in the animals receiving the lower dosages or in the oil-injected or untreated littermate controls. Animals in succeeding experiments were too young at autopsy to have spermatozoa in the epididymides or vasa deferentia.

### EFFECTS ON GONADOTROPIC POTENCY OF PITUITARY

At the autopsy of animals in Experiments I and II the pituitaries were fixed for histological study. However, when the animals in Experiments III and IV came to autopsy, the pituitary gonadotropic potencies were tested by the implant method. In

Experiment III there appeared to be a definite depression in gonadotropic potency of pituitaries of both injected animals, as judged by ovarian and uterine weight differences in the implanted mice. In Experiment IV no such depression was apparent.

### HISTOLOGICAL EFFECTS

Histological studies were made of certain organs from the experimental animals and their controls, to determine whether histological changes paralleled weight changes. A histological study of the testes was begun to determine what effect, if any, administration of progestin might have on sperm-head formation. Moore (1936) has shown that the time of appearance of sperm-heads in the testes of normal male rats of this colony is fixed at 33–35 days *post partum*. He found that this time of appearance was unaffected by injections of various gonadotropic preparations.

Experiments II, III, and IV were designed to test what effects administration of progestin might have on sperm-head appearance time. Two experiments (II and IV) were employed to test for any inhibitory action of the hormone on sperm-head formation within the dose range used. Injections were begun on day 25 and carried through day 39. Autopsy was on day 40. Oil-injected and normal littermate controls were run, and one normal control in each experiment was killed on day 33 to make sure that sperm-heads were not appearing extraordinarily early. It was decided that inhibition of sperm-head appearance would have to extend for about 5 days to be significant. Histological study showed that sperm-heads appeared by day 40 in all animals, including those treated with progestin. No sperm-heads were found in controls autopsied on day 33. Therefore, there was no apparent inhibitory effect from the dosages employed (0.5–3.0 Rb. U. daily) on the process of sperm-head formation.

Experiment III was planned to test for any accelerating action of progestin on sperm-head formation within the dose range employed. Injections ran from day 17 through day 31; autopsy was on day 32. Two dose-levels were used (0.5 and 1.0 Rb. U. daily). Oil-injected and normal littermate controls were run, and one normal control was allowed to live until day 36 to determine whether sperm-heads had formed by that time. Under these conditions there was no accelerating effect on sperm-head formation from administration of progestin. No sperm-heads were found in the testes of treated or control animals autopsied on day 32, whereas sperm-heads were found in the testes of the control autopsied on day 36. In the animal receiving 1 Rb. U. of progestin daily, not only was there no acceleration in the rate of sperm-head formation, but testis tubule diameters were definitely smaller than those of the normal littermates of the same age (ocular micrometer measurements). The testis weight was also far below normal (see Table 2).

The indications were, from these experiments, that progestin in the dosages employed had no effect on sperm-head appearance time but that testis weight and testis tubule diameter might be deleteriously affected.

Histological studies of other organs taken failed to reveal any striking differences correlated with progestin treatment. Organs studied included: (1) the sex accessories, such as ventral prostate, seminal vesicles, coagulating glands, periurethral tissue, Cowper's glands, and vas deferens; and (2) glands of internal secretion, such as adrenals, thymus, thyroids, and pituitary. Especial care was taken with the study of the pituitary, since Charipper (1934) had found what he called "pregnancy cells" in the pituitaries of normal and castrated male rats injected with 0.5 cc. of Lipolutin (Parke, Davis and

Company) for 15 days. According to Charipper, pregnancy cells are "large, ovoid, deeply eosin stained cells with homogeneous cytoplasm surrounding a usually eccentrically placed vesicular nucleus." Pituitaries of our experimental animals and their controls, and pituitaries of normal and pregnant female rats, were prepared according to Charipper's technique (Zenker fixation, Delafield's haematoxylin, and eosin). Eosinophilic cells were measured and studied for shape, for depth of stain, for homogeneity of cytoplasm, and for eccentricity of nucleus, without any special cell type having been found. Stein (1933, 1934) states that there are no special pregnancy cells in the rat. Other workers do not agree; nor do they agree on a definition of the term "pregnancy cell." Charipper (personal communication, 1937) now recognizes the error of the claims made in the 1934 paper.

### EFFECTS OF CORPUS LUTEUM HORMONE ON CASTRATED YOUNG MALE RATS

It has been shown above that certain sex accessories of normal young males underwent a slight hypertrophy correlated with the administration of progestin. This and other findings suggested that the injected material might have a slight androgen-like effect. The normal male—even though immature—is useless as a test animal for androgens, because the animal's own gonads may be elaborating physiologically similar substances. Therefore, it was deemed advisable to cease all experiments on normals and to try injections of corpus luteum hormone into castrates to determine whether our preparations had any androgenic potency.

### TREATMENTS

Littermates were castrated on the twenty-fifth day of life; and daily injections of corpus luteum hormone into some, of oil into others, were begun immediately. In all cases a normal littermate control was run. In most cases an uninjected castrated littermate control was run also. All treated animals and oil-injected controls were injected for 15 days, and all animals were autopsied on day 40. Progesterone was injected into four animals, in daily doses of 1, 2, 2, and 3 Rb. U. "Progestin II" was administered to one animal, 3 Rb. U. daily; and "Progestin III" was injected into one animal, also 3 Rb. U. daily. Six paired comparisons, therefore, were possible between treated castrates and their oil-injected castrated littermate controls.

### WEIGHT EFFECTS

Organ weight data have been analyzed on the basis of such paired comparisons. Because body weight was affected, a new analysis was made to take this factor into account. That is to say, each organ weight was converted into percentage of body weight, and the paired comparisons were made anew. A summary of the two analyses is presented in Table 3, together with results of a determination by Student's method of the statistical significance of the results. Despite the fact that dose-level and character of preparation were not constant for all six injectates, the six paired comparisons were considered as a unit.

It will be noted that sex accessories were, in general, heavier than the corresponding organs of the castrated controls. This was particularly true of the ventral prostate, preputial glands, and periurethral tissue. Coagulating glands were consistently heavier in the treated animals, whereas seminal vesicle weights showed such consistency only when analyzed on the basis of percentage of body weight, and Cowper's glands showed it not at all. Adrenal glands showed some suggestion of being lighter in the treated

animals, and the thymus gland was consistently lighter. Thyroids were, on the average, slightly heavier in the treated animals, and pituitary weight followed body weight closely.

TABLE 3

STATISTICAL STUDY OF THE EFFECTS OF CORPUS LUTEUM HORMONE ON THE ORGAN WEIGHTS OF CASTRATED YOUNG RATS

| Organ Weighed | On Basis of Absolute Weight | | | On Basis of Percentages of Body Weight | | |
|---|---|---|---|---|---|---|
| | Mean Δ* (Per Cent) | Range of Δ (Per Cent) | *P* | Mean Δ (Per Cent) | Range of Δ (Per Cent) | *P* |
| Body weight..... | − 9 | − 17 to − 3 | .013 | | | |
| Preputial glands... | + 82 | + 22 to + 124 | .008 | + 99 | + 42 to + 131 | .003 |
| Ventral prostate... | +684 | +300 to +1,329 | .005 | +746 | +380 to +1,383 | .003 |
| Periurethral tissue. | + 73 | + 12 to + 183 | .040 | + 90 | + 34 to + 228 | .034 |
| Coagulating glands | + 43 | + 4 to + 82 | .025 | + 57 | + 15 to + 109 | .013 |
| Seminal vesicles... | + 19 | − 2 to + 64 | .600 | + 31 | + 5 to + 90 | .065 |
| Cowper's glands... | + 27 | − 9 to + 55 | .053 | + 38 | − 6 to + 65 | .023 |
| Adrenal glands.... | − 18 | − 26 to − 11 | .001 | − 9 | − 24 to + 2 | .065 |
| Thymus gland..... | − 24 | − 42 to − 5 | .005 | − 17 | − 32 to − 2 | .010 |
| Thryoid glands.... | + 11 | − 5 to + 38 | .600 | + 23 | − 2 to + 66 | .069 |
| Pituitary gland.... | − 11 | − 23 to − 2 | .019 | − 1 | − 11 to + 12 | .940 |

* Δ = Difference between injectate and control.

Since *P* represented the probability coefficient, and a *P* of .05 or less was taken as evidence of statistical significance, it may be concluded that there was a significant weight increase in the ventral prostate, preputial glands, periurethral tissue, and coagulating glands of treated castrates. The weight increase in Cowper's glands was probably significant, whereas the weight increases of the seminal vesicles approached significance only when analyzed on the basis of percentage of body weight. The thymus was significantly lighter in treated animals; the adrenals were probably significantly affected; and the body weight proved significantly lower in treated castrates. Thyroids and pituitary seemed to be less affected.

As an additional check on the validity of the results and to show that such results could not be the effect of random sampling, a series of twelve paired comparisons was made between castrated littermates receiving identical treatment (oil injections). The reference animal of each pair was chosen at the time of castration (day 25), in order to have a fixed basis of comparison and to avoid the possibility of later selection of animals in such a fashion as to make plus and minus weight differences balance. Autopsy was on day 40. Treatment of the data by statistical methods identical with those used above showed that there was a strikingly close agreement between littermates treated identically, and that in only one instance, and by only one method of analysis, did a probability coefficient fall below .05.[4] Even this result fits well into the array of random significance figures which one would expect on the basis of chance.

[4] Seminal vesicle, on the basis of absolute weight (*P* was .015). On the basis of percentage of body weight the *P* was .174. The range of the other probability coefficients was, on the basis of absolute weight, .260–.759, and on the basis of percentage of body weight, .201–.915.

Moore (1937) has called attention to the androgen measure available in littermate-controlled experiments, made possible by comparing the growth of end-organs in treated castrates with that of normal littermate controls. To determine what accessory sex organs were kept at or near the normal weight-level by corpus luteum hormone in the doses employed, the organ weight data were analyzed on the basis of paired comparison between treated castrates and their normal littermate controls. The following general conclusions were drawn: (1) preputial glands, ventral prostate, and periurethral tissue weights were maintained at or near the organ weight levels of the normal controls; and (2) coagulating glands, seminal vesicles, and Cowper's glands were definitely lighter than the corresponding organs of the normal controls.

#### COMPARISON OF WEIGHT EFFECTS OF CORPUS LUTEUM HORMONE AND OTHER SEX HORMONES

A comparison of organ weight responses was made between progesterone-treated castrates and testosterone-treated castrates. A graded series of testosterone was used in an endeavor to find a dose range giving effects comparable to those following progesterone treatment. Dilute solutions of testosterone in sesame oil were injected subcutaneously in daily doses of 0.03 to 0.10 mg. from castration on day 25 through day 39. Autopsy was on day 40. Comparison of the pooled data with data in Table 3 showed that testosterone, even in 0.10-mg. doses, did not cause a weight increase for preputial glands or ventral prostate equal to that produced by 1–3 Rb. U. of corpus luteum hormone. On the other hand, testosterone in 0.08-mg. doses caused about the same periurethral tissue increases as did corpus luteum hormone in the doses used; and as little as 0.03 mg. of testosterone daily caused more growth of coagulating glands, seminal vesicles, and Cowper's glands than did the doses of corpus luteum hormone used. Pituitary and thymus weights were slightly depressed by all doses of testosterone used, though the statistical significance of this finding was not tested. Adrenal and thyroid weights were not affected in either direction by such doses of testosterone at this stage.

The comparative effects of testosterone and progesterone treatment were analyzed in another way: comparison of the organ weights of testosterone-treated castrates with those of their normal littermate controls made possible deductions as to how much testosterone was necessary to keep the sex accessories at, or near, the normal weight-level. It was concluded that at this age, about 0.06 mg. of testosterone performed this function for most of the castrate sex accessories. A dose-level below 0.06 mg. failed to do this, and higher dose-levels gave heavier-than-normal accessory weights. All sex accessories except the ventral prostate responded to testosterone by comparable weight increases for any one dose-level. Ventral prostate weight was comparatively lower than that of any other accessory. Comparison of this action of testosterone with the action of corpus luteum hormone (see above) showed great differences in the stimulating potencies of the two hormones on different end-organs.

A better method of analyzing such differences in the two hormones, perhaps, is the method of prostate–seminal vesicle ratios used by Moore and Price (1937), Moore (1937), and Moore and Price (1938). The prostate–seminal vesicle ratio is obtained by dividing the total weight of the entire prostate complex (ventral prostate, coagulating glands, and periurethral tissue) by the weight of the seminal vesicles. It has been shown that the ratio decreases with age in normal rats, until it reaches 1/1 at sexual maturity, and that both androsterone and testosterone are able to produce any normal ratio in castrates, provided proper doses are used. Low doses produce high ratios, and vice versa.

Data for progesterone-treated and testosterone-treated animals and their controls were analyzed to determine the prostate–seminal vesicle ratio. Five castrates treated with 0.06 mg. of testosterone daily gave ratios averaging 6.5, or about the average for the normal controls (6.76). On the other hand, six castrates treated with corpus luteum hormone gave ratios averaging 18.3. It is to be remembered that each of the treated groups differed from the normals hardly at all in the weights of three accessories (preputial glands, ventral prostate, and periurethral tissue), but that the seminal vesicles of the progesterone-treated castrates were decidedly below normal in weight. This suggests that the corpus luteum hormone may adequately stimulate certain male end-organs without materially affecting others, in a fashion which is at variance with the action of testosterone.

A comparison was made of organ weight responses of progesterone-treated castrates and of estrone-treated castrates of similar age. A graded series of estrone-treated castrates was run, sometimes using littermates of the progesterone-treated animals, sometimes using different litters. Dilute estrone was injected in oil in graded doses (2–20 international units daily) from day 25 to day 39, with autopsy on day 40. In general, small increases, not correlated with dose-level, were found in organ weights of preputial gland, ventral prostate, coagulating gland, and periurethral tissue of treated castrates, as compared with similar weights of oil-injected castrated controls. Seminal vesicle weights showed increases correlated with dose-level; and weights of Cowper's glands, adrenals, thymus, thyroids, and pituitary showed no definite trend correlated with treatment.

In experiments with all three hormones, untreated castrated controls showed organ weights entirely comparable with those of oil-injected castrated controls. However, for the sake of uniformity, oil-injected castrated controls have been used for comparison with treated animals.

### HISTOLOGICAL EFFECTS

Significant weight increases in certain accessory sex organs and weight decreases in the thymus of treated castrates led to the hypothesis that there might be an androgenic effect of corpus luteum hormone. Tissues taken at autopsy were divided into sex accessories and endocrine glands, and histological preparations were made. These two groups will be taken up in order.

#### SEX ACCESSORIES, OR ORGANS THAT MIGHT BE USED AS ANDROGEN INDICATORS

*The ventral prostate.*—The histology of the ventral prostate as an androgen indicator was worked out on the rat by Moore, Price, and Gallagher (1930); and a more careful investigation in young animals, both normal and castrated, was the work of Price (1936). She found that the normal prostate at 40 days is histologically similar to the adult gland, in that tubules are distended with secretion, gland cells are large and usually tall, and have "light areas" situated between the large basal round or oval nuclei and the free cell surfaces (see Fig. 1). These light areas are probably of significance in the physiology of secretion, for they are a constant feature of physiologically active cells, they disappear after castration, and they can be made to reappear with the administration of small doses of androgen. Consequently, they provide a sensitive test for the presence of androgens.

The prostates of animals castrated at 25 days and autopsied on day 40 showed striking differences from the normal picture (cf. Figs. 1 and 2). The tubular alveoli were smaller

and shorter. Tubular diameters were extremely reduced, as was also the amount of secretion within the tubule. Each gland cell was small, and practically cuboidal, with the nucleus near the center. Nuclei were smaller and more deeply stained. There was a relative (but probably not a true) increase in the intertubular connective tissue. Despite the fact that Price (1936) states that light areas persist in 30–40-day rats castrated during the first week of life, no light areas were found in animals castrated on day 25 and autopsied on day 40. Essentially the same thing was found by Greene, Burrill, and Ivy (1939).

In each comparison of normal control, castrated control, and corpus luteum hormone treated castrate, the ventral prostate of the treated castrate resembled, in all particulars, that of the normal control rat (cf. Figs. 1, 2, and 3). The tubules were distended with secretion; tubular diameters were fully as large as normal; and the gland cells were large, high columnar cells with basal nuclei. The nuclei were large and lightly stained. The proportion of tubular tissue and intertubular connective tissue was normal. Finally, light areas were found in the gland-cell cytoplasm between the nuclei and free cell borders.

Thus, it is apparent that the injected material, either corpus luteum extract or synthetic progesterone, acted as an androgen in building and maintaining the prostate in the normal secretory state.

*Cowper's gland.*—Heller (1930, 1932) worked out the histology of Cowper's gland as an androgen indicator. He says that by 40 days "the gland appears to have entered definitely upon its secretory activity." In adults, castration atrophy appears by 10 days, and is well advanced by 20 days, after castration.

In the normal 40-day-old rat Cowper's gland consisted of compound acini emptying into a central lumen. The entire gland was covered with a fibrous and muscular capsule. The central cavity and the gland duct were lined with a stratified epithelium. The acini were lined with tall columnar cells having small basally located, flattened nuclei, which were stained deeply with haematoxylin. The cytoplasm was very homogeneous and granular.

In rats castrated on day 25 and autopsied on day 40 Cowper's gland was much smaller than in the normal control. Considerable secretion still could be found in the central cavity, and there was a relatively greater amount of interacinous connective tissue and capsular material. Little secretory tissue was present. The few small acini were crowded between capsular connective tissue and the wall of the central cavity. Gland-cell height was much below that of the normal. Gland-cell nuclei were rounder and larger, less densely stained than in the normal. The condition was comparable to that of a much longer castration period in the adult (between 20 and 90 days).

In Cowper's glands of treated castrates the condition was intermediate between that described for the normal and for the castrated control. The secretory cells were higher, the acini were more numerous, and the interacinous connective tissue was relatively less prominent than in the oil-injected castrated control. Ocular-micrometer measurements showed that cell heights and acinar diameters were intermediate between those of the castrate and those of the normal control.

*The vas deferens.*—Vatna (1930) has worked out the histology and cytology of the vas deferens as an androgen indicator. Many changes due to castration were observed. He states that gross size is reduced, owing to a regression of the tunica muscularis. The amount of secretion within the lumen is diminished; and there is a reduction in epithelial cell height, a stratification of nuclei in the epithelial layer, obliteration of epithelial cell

walls, and a more or less complete loss of cilia-like structures from the epithelial surface. Where the "cilia" are not completely gone, they are short, matted, and disarranged.

In the castrate-control vas deferens at 40 days only some of the changes mentioned by Vatna were found. Obliteration of epithelial cell walls could not be seen in all, nor was there a difference in the amount of secretion within the lumen. Perhaps the more clearly defined changes from the normal in these animals were (1) reduction of the cilia-like processes, (2) reduction in gross size, and (3) apparent stratification of epithelial nuclei. Nuclei in the epithelial cells of castrates tended to be more basal than is normal.

Corpus luteum hormone treated castrates showed a histology and cytology of the vas deferens entirely comparable to the normal control. Lumina were large, and the epithelial cells retained their size and their long cilia-like processes. Gross cross-sectional size of the vas deferens was essentially that of the normal control. Nuclei of the epithelial cells were usually located about two-thirds of the distance from the free border of the cell to its base, as was the case in the normal controls.

*Seminal vesicle.*—The seminal vesicle cytology test has been suggested as a male hormone indicator by several workers. As worked out by Moore, Hughes, and Gallagher (1930), a positive test depends on the presence of secretion granules in sections of the seminal vesicle stained with Ehrlich's haematoyxlin. Castration causes a disappearance of secretion granules from the adult seminal vesicle within 48 hours.

At 40 days the normal controls of the experimental series were at the beginning of their adult secretory state. Epithelial cells were not so high as in the adult, and secretion granules with halos were not so prominent or so frequent as in the adult. Secretion granules were, however, undoubtedly present by 40 days (Price, 1936, says they appear by day 36), and secretion could be seen in the lumen of the gland.

The condition of the castrate-control seminal vesicle at 40 days was distinctly different from that of the normal control. The cavity of the gland was much smaller and was not filled with a stainable secretion. The blind out-pouchings of the central cavity were not extensive. Secretory epithelium showed a severe castration atrophy. The cells were low, scarcely higher than the diameter of the nucleus; and no secretion granules were present.

Treated castrates showed essentially the same seminal vesicle histology and cytology as did the castrated controls. Epithelial cells were low, and there was practically no cytoplasm distal to the nucleus. In one case only was stainable secretion found in such a seminal vesicle. Search revealed a few secretion granules in a consistently higher epithelium. This was in RE175, an animal which received 3 Rb. U. of "Progestin III" daily, in dilute solution. Seminal vesicle weight increase was also the most pronounced (+90 per cent) of any animal in the series.

*Middle lobe of the prostate* (*periurethral tissue*).—Korenchevsky and Dennison (1935) have divided this prostatic tissue into three general subdivisions: lateral, dorsal, and central lobes. Moore (1939) mentions lateral and dorsal subdivisions only. Histological study was confined to the lateral subdivision. Acinar structure somewhat different from that of the ventral prostate was found. Cell heights of the normal and castrate gland did not differ appreciably, but acinar diameters decreased following castration. Similarly, effects of corpus luteum hormone were not on cell height, but rather on acinar diameters. Acinar diameters in the lateral subdivision of the prostate of treated animals averaged well above those of both the castrate and the normal control.

*The coagulating gland.*—Moore, Price, and Gallagher (1930) found that in the adult

the coagulating gland responds to sex hormone states and is an additional, though less satisfactory, androgen indicator. Response to androgens is merely one of epithelial cell size and acinar size.

A detailed comparison between normal and castrated controls of the experimental series, autopsied on day 40, failed to reveal any histological basis in the coagulating gland for distinguishing between the two catagories of animals. Cell height measurements and acinar diameter measurements were not essentially different in the normal, castrated control or in castrated treated animals.

*The preputial glands.*—Preputial glands of adult rats respond to castration by certain well-defined histological changes, according to Korenchevsky and Dennison (1935). These are reductions in the number and size of the cells and alveoli. Cells become atrophic and more irregular. Vacuolization of the cytoplasm is coarser. Pyknotic nuclei are more numerous, and nuclei are packed closer together. Canals and ducts are smaller, are less ramified, and contain less secretion.

None of these changes were found in castrated controls of the experimental series, whether because of early age at castration and autopsy, because of short castration time, or because of some other factors we do not know. Neither was any histological change noted in the preputial glands of treated castrates.

### ENDOCRINE GLANDS

Histological preparations of the adrenal gland, thymus, and thyroid were studied; but no striking changes attributable to either castration or corpus luteum hormone treatment were evident. Mammary gland and pituitary gland preparations have not been made.

A summary of the histological effects of corpus luteum hormone on castrates is presented in Table 4.

TABLE 4

| Organ | Effects of Castration from Day 25 to Day 40 | Effects of Hormone (1–3 Rb. U. Daily) into Castrates from Day 25 to Day 40 |
|---|---|---|
| Ventral prostate | Castration atrophy | Prevention of castration atrophy; light areas present |
| Vas deferens | Castration atrophy | Prevention of castration atrophy |
| Cowper's glands | Castration atrophy | Amelioration of castration atrophy |
| Seminal vesicles | Castration atrophy | Slight repair in one case only |
| Middle prostate | Decrease in tubular diameters | Tubular diameters larger than in castrate or normal controls |
| Coagulating glands | No effects | No effects |
| Preputial glands | No effects | No effects |
| Adrenal glands | No effects | No effects |
| Thymus gland | No effects | No effects |
| Thyroid glands | No effects | No effects |

### EFFECTS OF IMPLANTS OF PITUITARIES INTO MICE

Three experimental series of animals had the pituitary gonadotropic potency assayed on 21-day female mice. No depression or augmentation of gonadotropic potency was observed which could be correlated with corpus luteum hormone treatment.

## Discussion

It has been seen in the preceding section that certain tests on male rats indicated a weak androgenic activity of corpus luteum extracts and of synthetic progesterone. A critical search of the literature published prior to the presentation of the preliminary report of this work failed to reveal claims of androgenic effects of corpus luteum hormone in males. In fact, most of the workers dealing with the subject of corpus luteum hormone effects in males have been unable to demonstrate any outstanding effects of hormone administration. Greene, Burrill, and Ivy (1939) have been able to confirm and to extend some of the conclusions presented in the preliminary report of this work.

All articles dealing with possible effects of corpus luteum hormone on male individuals may be classified into one of three general groups: (1) those claiming no effects, (2) those claiming effects which have been brought into question by later work, and (3) those claiming effects which have not been questioned. Of the first group, the work of Fels (1935), of Albrieux, Buño, Engel, and Morató-Manaro (1936), of Fels (1936, 1937), and of Korenchevsky (1937) may be mentioned. As examples of the second group, the papers of Charipper (1934) and of Clauberg and Breipohl (1935) are cases in point. These authors claimed that corpus luteum extracts correct castration cells in the pituitaries of castrated rats. Fels (1935) and Hohlweg (1935), using larger doses of crystalline progesterone, failed to confirm this observation. They suggested that the results of the original workers may have been due to estrogenic impurities in their preparations. Charipper (1934) claimed also that "pregnancy cells" can be formed in the pituitaries of either normal or castrated male rats by administration of corpus luteum extracts. The work of Stein (1933, 1934) and the admission of Charipper (personal communication, 1937), already noted, dismiss this assertion. Examples of effects which have been reported but not questioned are the following: (1) Gardner and Hill (1936) have been able to produce mammary gland growth in normal and castrated male mice by corpus luteum treatment; (2) Zuckerman and Parkes (1936) found that the administration of progesterone to male monkeys causes no change in the prostate or other sex accessories but does tend to correct the pathology of the lining of the uterus masculinus which has resulted from injected estrone; (3) Burrows (1936) claimed that progesterone protects normal male mice, but not castrates, from the harmful effects of applied estrone; and (4) Nelson (1936, 1937) found that corpus luteum hormone is as effective as androgens in maintaining spermatogenesis in hypophysectomized rats but that no androgenic effects are evinced either by the injected material directly or by indirect stimulation to production of testicular internal secretion.

Whereas there were no previous claims in the literature that corpus luteum extracts or progesterone in males act as androgens, there were certain indications that such might be the case and certain findings which can be explained better, perhaps, by such an assumption. Steinach and Kun, as early as 1931, found that in the female guinea pig transformation of the clitoris into a penis-like organ results from any one of three kinds of treatment, namely, (1) X-irradiation of the ovaries, followed by luteinization; (2) administration of gonadotropic extract, followed by luteinization; or (3) administration of luteal extracts (into spayed or normal females). In all cases the level of corpus luteum hormone in the blood would be raised. Hill (1937*a*, 1937*b*, and 1937*c*) found that ovarian grafts in the ears of castrated male mice luteinize and that, where such grafts are present, the castrate accessories are repaired. Price (personal communication, 1937)

obtained "functional appearing" ventral prostate grafts when the hosts were either normal virgin female rats or female rats kept pregnant. Grafts are larger, on the average, in the latter hosts. Parkes (1937), using a very sensitive comb-growth test and extraction methods similar to our own for extracting corpus luteum hormone, found that certain ovarian extracts are slightly but definitely androgenic. Finally, as very good evidence, Steinach and Kun (1931) administered luteal extracts to young castrated male rats and produced normal-looking penis development. They thought that the corpus luteum produces not one hormone but two, namely, (1) a hormone for the production of a progravid uterine endometrium, and (2) a hormone identical with testis hormone. All these evidences fit into the general picture and are unified by the results of the present experiments.

A study of comparative effects of progesterone and testosterone suggests that the two may differ somewhat in their physiological expression, although both may act as androgens. A great deal more evidence is needed to corroborate this suggestion, but it does present some interesting implications. That androgens may differ among themselves in relative potency, and that a single androgen may show threshold differences with regard to different end-organs, is well known. It has also been strongly suggested (see Deanesly and Parkes, 1936; Korenchevsky, Dennison, and Brovsin, 1936; and Tschopp, 1936; and their citations) that androgens may differ among themselves as to their relative threshold differences—that is, a certain hormone may exhibit a "preferential" action on an end-organ out of all proportion to its effects on other end-organs, as compared with other androgens. Moore (Moore and Price, 1937; Moore, 1937; and Moore and Price, 1938) has brought this idea into question with his work on prostate–seminal vesicle ratios, and the problem is still unsettled. It is very certain that progesterone exhibits threshold differences for different end-organs in the castrated young male rat. Whether such differences are comparable with those exhibited by testosterone or are actually preferential for prostate tissue over and above those of testosterone, as they seem to be, remains for further work to decide.

There remains the question, still unanswered, as to whether progesterone acts as an androgen per se or is changed in the body by relatively simple chemical reorganization into an androgen. It is true that progesterone is more closely similar chemically to certain androgens, i.e., testosterone and androstenedione, than are these hormones to other androgens. The simplest change of progesterone to any of the known androgens would probably be to testosterone. However, since the physiological expression of the two (testosterone and progesterone) seems to be dissimilar, it is unlikely that this change takes place. It seems quite as possible that the progesterone acts per se as a weak androgen.

It is not unlikely that still other weight responses and histological changes may be found to result from progesterone treatment when more extensive work is done, using more animals and larger doses of the hormone. When considered on the basis of percentage of body weight, the adrenals and thyroids of treated castrates showed weight decreases approaching a statistically significant value. Also, it is possible that with larger doses the seminal vesicles may be affected in the same manner as are the other sex accessories.

That sex accessories, which would be expected to act in similar fashion under sex hormone stimulation, showed, in general, weight increases, whereas other organs showed no change in weight and still others showed weight decreases, adds to the dependence to

be put in the results. Confirmation is to be had in histological changes correlated with treatment. All positive effects indicate a weak androgen-like activity of progesterone.

That the effects seen are due to progesterone alone, and not to any other substance, seems indicated by the following facts: (1) controls injected with plain oil showed none of the effects; (2) all extracts used were treated to remove estrogens; (3) most of the work on castrates was done using synthetic crystalline progesterone, both the extracts and pure progesterone giving the same results; and (4) the comparison of progesterone effects with effects of estrone showed a decided qualitative difference in the two hormones.

Korenchevsky (1937) has advanced the idea that sex hormones can be classified into one of three general categories, namely, (1) monosexual, (2) partly bisexual, and (3) true bisexual hormones. Most hormones fall into the bisexual groups. In fact, progesterone alone was placed in the monosexual category as a purely female hormone. This idea has been controverted by the present experiments, and it may now be said that we know of no purely monosexual hormone.

## Summary

1. Results of administration of corpus luteum extracts to normal young male rats suggested a weak androgenic action of the preparations.

2. Within the dose ranges employed ($\frac{1}{2}$–3 Rb. U. [Corner] daily for 15 days) there was no slowing or acceleration of the rate of sperm-head formation due to the injected corpus luteum extracts.

3. Progesterone or corpus luteum extracts injected (1–3 Rb. U. [Corner] daily for 15 days) into castrated young male rats caused (1) a significant weight increase for preputial glands, ventral prostate, periurethral tissue, coagulating glands, and possibly Cowper's glands; (2) a significant weight decrease for body weight and thymus gland; and (3) no decided weight change for seminal vesicles, adrenals, thyroids, and pituitary.

4. Difference in grade of weight response of the various sex accessories to progesterone may be attributed to a threshold phenomenon.

5. Comparison of weight effects of progesterone and testosterone suggests a difference between the two with respect to relative thresholds for prostatic tissue and the seminal vesicle.

6. Castrated sex accessories which responded histologically to progesterone and to corpus luteum extracts by amelioration of castration atrophy were, in order of decreasing amelioration: ventral prostate, vas deferens, lateral lobe of middle prostate, Cowper's glands, and seminal vesicles. Accessories which responded histologically neither to castration nor to injections were the coagulating glands and preputial glands. Adrenals, thymus, and thyroids showed no histological changes to castration or to corpus luteum hormone injections under the experimental conditions.

7. No difference in pituitary gonadotropic potency was observed between injected castrates and their controls.

8. All positive effects of progesterone and corpus luteum extracts in young male rats, castrated or normal, pointed to an androgenic potency of these preparations.

9. Since results of progesterone and corpus luteum extracts on castrates were entirely similar, and since injections of estrone gave very dissimilar results it may safely be concluded that all results were due to the administration of the corpus luteum hormone and not to any other hormone or impurity.

10. Prior to these experiments progesterone was the only sex hormone for which bisexual effects had not been demonstrated. Now it has been shown to have effects on accessory sex organs in the male.

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PLATE I

FIG. 1

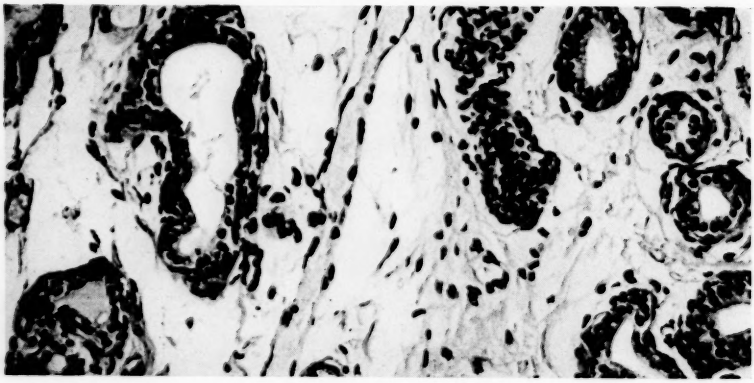

FIG. 2

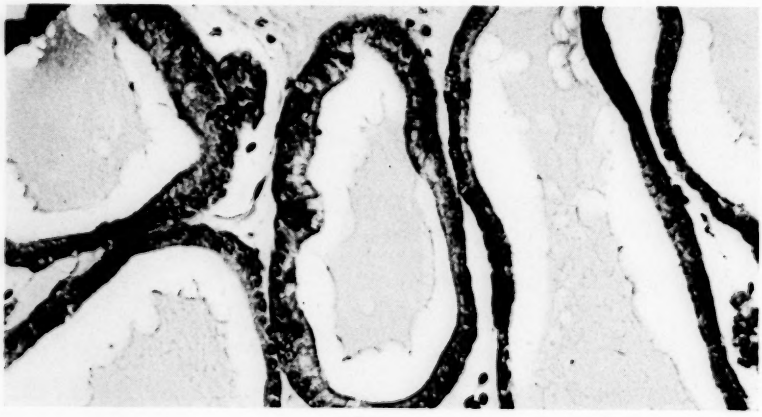

FIG. 3

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## PLATE I

FIG. 1.—Section of a normal ventral prostate at 40 days. ×260.

FIG. 2.—Section of the ventral prostate of a littermate, castrated from day 25 to day 40. ×260.

FIG. 3.—Section of the ventral prostate of a littermate castrated, and injected with 2 mg. of progesterone daily, from day 25 to day 40. ×260.

# EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE ON THE THYROID AND ADRENAL GLANDS OF THE GROUND SQUIRREL, CITELLUS TRIDECEMLINEATUS[1]

MOSES ZALESKY AND L. J. WELLS
Hull and Whitman Zoölogical Laboratories, University of Chicago, and
Department of Anatomy, University of Missouri

IN 1931 a group of workers at the zoölogical laboratories of the University of Chicago, including the writers of the present paper, began a systematic investigation of the reproductive cycle of the thirteen-lined ground squirrel, *Citellus tridecemlineatus*, as well as of the factors underlying and influencing this reproductive cycle. The results of this investigation were presented, in condensed form, in a general report (Moore *et al.*, 1934) and were subsequently elaborated by Wells (1935*a*) for the male and by Simmons (1934) for the female. The sexual cycle of *C. tridecemlineatus* was described, and evidence was presented that among the endocrine factors involved in the manifestation of reproductive periodicity in this wild, annual-breeding mammal the hypophysis played a significant role. In an attempt to analyze the environmental conditions which in turn influence the hypophysis, the effect of low environmental temperatures upon the animal, among other factors, was investigated. Peculiarly enough, it was found that animals of either sex placed during the season of breeding capacity (April–May) in a cold room with a constant temperature of +4° C. tended to remain on a high level of sexual development so long as they were exposed to the colder environment, whereas normally they undergo sexual retrogression during the summer. This effect of low environmental temperature appeared striking and stimulating to further investigation. Thus, the possibility of observing corresponding effects of low environmental temperatures upon the thyroid and adrenal glands suggested itself as an object of especial interest in the study of endocrine interrelationships, particularly in view of the fact that data on the seasonal cycle in the thyroid and adrenal glands of *C. tridecemlineatus* have previously been obtained by the senior author (1934 and 1935, respectively) and were thus available for comparison.

## MATERIALS AND METHODS

This investigation was conducted on 39 males (24 experimental and 15 controls) and 26 females (13 experimental and 13 controls). The experimental animals were captured in the field during April and early in May, i.e., during the period of high sexual development and breeding capacity, and placed immediately thereafter in an electrically refrigerated room in which the temperature was maintained thermostatically at +4° C. with a high degree of constancy. The control animals were also collected in the field during the same season and kept in the laboratory at room temperature. The animals

[1] The writers wish to acknowledge their indebtedness to Dr. Carl R. Moore for his helpful guidance and critical reading of the manuscript; to Dr. W. C. Allee for placing at the writers' disposal the cold-room facilities of the Whitman Zoölogical Laboratory; and to Miss Ruth Berman for her valuable technical assistance.

of the cold room were isolated from each other in small individual cages, so that they were unable either to cuddle together and thus reduce the loss of their body heat or to devour their hibernating cagemates. The controls were caged in groups of 4–6. The food of both the experimental animals and the controls consisted of mixed grain with added "rabbit chow checkers." These "checkers" are prepared by the Purina Company and contain 16.45 per cent of protein. Ground squirrels seem to thrive on this diet and show evidence of good health and satisfactory physical condition. The relative humidity of the cold room[2] was 62.7, whereas in the laboratory it registered only 38.2. It is doubtful, however, whether the difference in humidity influenced appreciably the results of the experiment, since animals previously kept under various conditions of humidity had not shown any observable physiological or developmental differences.

To exclude possible effects of darkness, 14 males and 8 females of the experimental lot were subjected to ultra-violet radiation for 4 hours daily for periods ranging from 28 days to $7\frac{3}{4}$ months in the otherwise dark cold room. Two Westinghouse mercury-vapor lamps, type g-5, with an output of 15.23 microwatts per square centimeter,[3] were used. All experimental animals were sacrificed on the day of their removal from the cold room, along with several controls.

At autopsy the reproductive organs, the thyroids and the adrenals were removed, weighed fresh, and prepared for histological study. Usually one thyroid and one adrenal from each animal were fixed in Bouin's fluid, while for the remaining thyroid and adrenal Bensley's formalin-Zenker was used as fixative.

Omission of 95 per cent and absolute alcohols from the dehydration process of the thyroid and adrenal and the substitution of aniline oil for these alcohols ($\frac{2}{3}$ of 70 per cent alcohol $+\frac{1}{3}$ of aniline oil for 1–2 hours, $\frac{2}{3}$ of aniline oil $+\frac{1}{3}$ of 95 per cent alcohol for 1–2 hours, and then pure aniline for 1–2 hours) were found helpful for satisfactory sectioning. The tissues were imbedded in a mixture of 60° paraffin, 1 per cent rubber, and 5 per cent Bayberry wax, and sectioned at 4–5 $\mu$. The staining was done chiefly in Krichesky's modification of the triple Mallory mixture.

For the determination of the relative width of the zones in the adrenal cortex, an ocular micrometer was used.

## RESULTS

The prolonged exposure of the thirteen-lined ground squirrel to a practically constant temperature of +4° C. caused the animal to exhibit hibernation tendencies. However, in no case was hibernation sustained and uninterrupted. Various degrees of intermittency in hibernation were observed. Some animals were usually in torpor when periodic observations were made, while others were never seen in the hibernating state.

Loss of body weight was, as is to be expected, directly proportional to the relative length of time spent by a given animal in hibernation. The mean body weight of 24 experimental adult males was 178 gm., while in 15 comparable control adult males it amounted to 225 gm. (Table 1). The average body weight of the 13 experimental females (all adult) was 169 gm., whereas in the comparable controls of this sex the corresponding average was 198 gm. (Table 2).

The effects on the reproductive system of prolonged exposure of males of the thirteen-

[2] A Tycos wet-and-dry-bulb autohigrometer was used.

[3] At a point on the axis of the aluminium reflector 1 meter from the source.

## TABLE 1

### COMPARISON OF BODY WEIGHT, SEXUAL CONDITION, AND WEIGHT OF THE THYROID AND ADRENAL GLANDS IN COLD-ROOM AND CONTROL MALES

#### A. EXPERIMENTAL MALES

| Number of Animal | Placed in Cold Room | Sacrificed | Body Weight (Gm.) | Weight of Testis (Gm.) | Spermatozoa in Epididymis* | Weight of Thyroids (Mg.) | Weight of Adrenals (Mg.) |
|---|---|---|---|---|---|---|---|
| 1024 | 4-13-35 | 8-27-35 | 160 | 0.7250 | ++++ | ..... | ..... |
| 1025 | 5-27-35 | 8-27-35 | 184 | 0.5074 | ++++ | ..... | ..... |
| 1085 | 4-13-35 | 11- 6-35 | 135 | 0.6872 | ++++ | ..... | ..... |
| 1086 | 4-13-35 | 11- 6-35 | 160 | 0.6000 | ++++ | ..... | ..... |
| 1087 | 4-13-35 | 11- 6-35 | 165 | 0.5212 | ++++ | ..... | ..... |
| 1088 | 4-13-35 | 11- 6-35 | 139 | 0.5108 | ++++ | ..... | ..... |
| 1170 | 4-13-35 | 4-22-36 | 170 | 0.6470 | ++++ | 22.4 | 21.0 |
| 1171 | 4-13-35 | 4-22-36 | 180 | 0.4950 | ++++ | 43.0 | 22.2 |
| 1268 | 4-18-36 | 8-30-36 | 211 | 1.0190 | ++++ | 22.6 | 31.2 |
| 1269 | 4-18-36 | 8-30-36 | 195 | 0.8700 | ++++ | 22.0 | 33.8 |
| 1272 | 4-18-36 | 9-29-36 | 180 | 0.8360 | ++++ | 17.0 | 40.0 |
| 1273 | 4-18-36 | 9-29-36 | 160 | 0.7650 | ++++ | 26.2 | 22.0 |
| 1289 | 4-18-36 | 10-31-36 | 211 | 0.7220 | ++++ | 50.0 | 43.0 |
| 1290 | 4-18-36 | 10-31-36 | 190 | 0.5870 | ++++ | 26.0 | 16.0 |
| 1292 | 4-18-36 | 12- 2-36 | 175 | 0.7490 | ++++ | 39.6 | 18.8 |
| 1294 | 4-18-36 | 12-24-36 | 200 | 0.6745 | ++++ | 29.0 | 21.0 |
| 1295 | 4-18-36 | 12-24-36 | 180 | 0.3170 | 0 | 20.0 | 32.0 |
| 1322 | 4-18-36 | 1-29-37 | 190 | 0.5874 | ++++ | 39.7 | 26.5 |
| 1362 | 4-18-36 | 2-25-37 | 178 | 0.6144 | ++++ | 25.6 | 21.1 |
| 1377 | 4-18-36 | 4-23-37 | 187 | 0.6578 | ++++ | 19.4 | 31.6 |
| 1378 | 4-18-36 | 4-23-37 | 187 | 0.6273 | ++++ | 20.3 | 19.0 |
| 1379 | 4-18-36 | 4-23-37 | 190 | 0.6195 | ++++ | 17.4 | 14.3 |
| 1400 | 5-11-37 | 11-19-37 | 170 | 0.5138 | ++++ | 53.8 | 21.6 |
| 1401 | 5-11-37 | 11-19-37 | 155 | 0.4351 | ++++ | 17.8 | 21.0 |
| Mean | ..... | ..... | 178 | 0.6370 | ..... | 27.3 | 25.9 |

#### B. CONTROL MALES

| Number of Animal | Sacrificed | Body Weight (Gm.) | Weight of Testis (Gm.) | Spermatozoa in Epididymis* | Weight of Thyroids (Mg.) | Weight of Adrenals (Mg.) |
|---|---|---|---|---|---|---|
| 1211 | 7-18-36 | 295 | 0.1412 | 0 | ..... | ..... |
| 1212 | 7-18-36 | 235 | 0.1192 | 0 | ..... | ..... |
| 1215 | 8-30-36 | 210 | 0.1800 | 0 | 35.9 | 19.4 |
| 1217 | 8-30-36 | 275 | 0.1182 | 0 | 24.5 | 23.7 |
| 1218 | 8-30-36 | 205 | 0.1574 | 0 | 26.1 | 27.8 |
| 1220 | 8-30-36 | 195 | 0.1414 | 0 | 15.3 | 24.4 |
| 1221 | 8-30-36 | 235 | 0.2558 | 0 | 17.0 | 29.5 |
| 1270 | 8-30-36 | 342 | 0.2302 | 0 | 12.4 | 21.8 |
| 1271 | 8-30-36 | 190 | 0.1268 | 0 | ..... | ..... |
| 1274 | 10-14-36 | 205 | 0.2450 | 0 | ..... | ..... |
| 1275 | 10-14-36 | 120 | 0.1200 | 0 | ..... | ..... |
| 1288 | 10-31-36 | 215 | 0.3160 | 0 | 24.4 | 54.2 |
| 1291 | 12- 2-36 | 258 | 0.2940 | 0 | 21.8 | 27.8 |
| 1293 | 12-24-36 | 205 | 0.5850 | 0 | 22.0 | 21.0 |
| 1321 | 1-29-37 | 190 | 1.1720 | ++ | 21.5 | 26.8 |
| Mean | ..... | 225 | 0.2801 | ..... | 22.1 | 27.6 |

* Estimate of quantity and motility of spermatozoa in the epididymis ranges from 0 (spermatozoa lacking) to ++++ (spermatozoa abundant, vigorously motile).

lined ground squirrel to low environmental temperature have been reported and described by the writers elsewhere (Wells, 1935*b*; Wells and Zalesky, 1940). For the pur-

TABLE 2

COMPARISON OF BODY WEIGHT, SEXUAL CONDITION, AND WEIGHT OF THE THYROID AND ADRENAL GLANDS IN COLD-ROOM AND CONTROL FEMALES

A. EXPERIMENTAL FEMALES

| Number of Animal | Placed in Cold Room | Sacrificed | Body Weight (Gm.) | Weight of Ovaries (Mg.) | Weight of Uterus (Mg.) | Condition of Vaginal Membrane | Weight of Thyroids (Mg.) | Weight of Adrenals (Mg.) |
|---|---|---|---|---|---|---|---|---|
| B- 3..... | 5-3-34 | 11-23-35 | 176 | 31.5 | 2,320.0 | open | 37.4 | 39.8 |
| B-64..... | 5-6-36 | 12-16-36 | 168 | 14.4 | 828.0 | open | 30.8 | 28.6 |
| B-65..... | 5-6-36 | 12-16-36 | 187 | 38.0 | 402.0 | open | 35.7 | 20.0 |
| B-66..... | 5-6-36 | 12-16-36 | 187 | 28.8 | 833.0 | open | 32.0 | 14.0 |
| B-68..... | 5-6-36 | 12-17-36 | 165 | 35.0 | 455.0 | open | 22.8 | 41.0 |
| B-69..... | 5-6-36 | 12-17-36 | 193 | 15.0 | 330.0 | open | 39.8 | 12.0 |
| B-70..... | 5-6-36 | 12-17-36 | 205 | 33.8 | 525.2 | open | 36.6 | 37.5 |
| B-71..... | 5-6-36 | 12-17-36 | 189 | 27.4 | 604.6 | open | 34.0 | 20.0 |
| B-72..... | 5-6-36 | 12-17-36 | 202 | 31.0 | 667.0 | open | 30.4 | 14.6 |
| B-94..... | 5-3-37 | 12- 1-37 | 115 | 12.2 | 541.8 | open | 35.8 | 21.8 |
| B-95..... | 5-3-37 | 12- 1-37 | 149 | 16.8 | 584.6 | open | 40.2 | 21.0 |
| B-97..... | 5-3-37 | 12- 4-37 | 117 | 15.6 | 335.8 | open | 37.0 | 26.0 |
| B-98..... | 5-3-37 | 12- 4-37 | 140 | 25.0 | 781.0 | open | 30.3 | 27.0 |
| Mean.. | ..... | ..... | 169 | 25.0 | 708.4 | ..... | 34.8 | 24.9 |

B. CONTROL FEMALES

| Number of Animal | Sacrificed | Body Weight (Gm.) | Weight of Ovaries (Mg.) | Weight of Uterus (Mg.) | Condition of Vaginal Membrane | Weight of Thyroids (Mg.) | Weight of Adrenals (Mg.) |
|---|---|---|---|---|---|---|---|
| B-63..... | 12-16-36 | 180 | 16.0 | 242.0 | closed | 24.9 | 12.4 |
| B-67..... | 12-16-36 | 212 | 20.0 | 118.0 | closed | 21.0 | 19.0 |
| B-73..... | 12-17-36 | 205 | 20.2 | 155.8 | closed | 28.3 | 16.8 |
| B-74..... | 12-17-36 | 188 | 13.2 | 90.8 | closed | 23.8 | 11.0 |
| B-75..... | 12-17-36 | 190 | 15.0 | 61.0 | closed | 24.6 | 11.8 |
| B-76..... | 12-18-36 | 167 | 16.6 | 409.4 | closed | 36.0 | 20.0 |
| B-77 ..... | 12-18-36 | 205 | 17.2 | 226.8 | closed | 23.8 | 11.4 |
| B-78..... | 12-18-36 | 216 | 21.4 | 128.6 | closed | 24.2 | 16.2 |
| B-93..... | 12- 1-37 | 164 | 13.6 | 120.8 | closed | 27.0 | 17.6 |
| B-96..... | 12- 4-37 | 200 | 13.6 | 114.6 | closed | 29.6 | 20.8 |
| 895..... | 12- 2-34 | 252 | 17.7 | 144.9 | closed | 11.8 | 20.0 |
| 892..... | 11-16-34 | 148 | 14.2 | 116.2 | closed | 10.4 | 17.6 |
| 893..... | 11-16-34 | 129 | 13.4 | 110.0 | closed | 21.2 | 11.0 |
| Mean.. | ..... | 198 | 16.3 | 156.8 | ..... | 23.6 | 15.8 |

pose of the present investigation it will suffice to note that the males subjected to low environmental temperatures did not undergo sexual regression, which normally takes

place in the male during the early summer and which persists until new seasonal development is initiated in December or January.

Also in the female, as previously reported (Moore *et al.*, 1934; Simmons, 1934), corresponding effects were observed. Females placed in the cold room early in May, i.e., at or near oestrum, exhibited at autopsy in December a state of high sexual development, whereas in nature or in the laboratory they are sexually regressed from August to February.

The mean weight of the thyroid (Table 1)[4] in the experimental group of adult males (27.3 gm.) shows an increase of 24 per cent over the corresponding average in the control lot (22.1 mg.). This increase, however, is statistically not very significant, and the number of males whose thyroid weight was available was small (18 experimental and 10 controls). The establishment of the possible significance of the increase in weight of the thyroid gland under conditions of low environmental temperatures would require a larger series, in view of the normally wide range of variation in the weight of the thyroid gland of the ground squirrel.

Histologically, nothing could be observed to justify a significant increase of weight in the thyroid gland of the male ground squirrel under the influence of low environmental temperature. The thyroid of the experimental animals resembled in every detail the thyroid of the controls, except perhaps for the tendency toward a larger number of small follicles displayed by some experimental animals.

In so far as could be determined from histological study, the glands of the experimental males were essentially in the same phases of activity as those of the laboratory controls. They were of two types, either one or the other predominating within the limits of a given gland. Most common was the "resting" gland, with its low epithelium, compressed, elliptical nuclei, distended follicles, and dense colloid. Occasionally the "storing" gland was encountered. In this type of gland the epithelium is largely low cuboidal or cuboidal, the nuclei of the epithelial cells are spheroid, the apical portion of the epithelial cells contains colloid floccules, and the colloid is usually less dense than in the "resting" gland. The "storing" gland is no doubt secreting through the apical pole of the cell into the lumen of the follicle, thus storing colloid for the future need of the organism. Vacuoles of resorption are exceedingly rare in either the "resting" or the "storing" thyroid. Whenever found, they are located in one or several of the smaller central follicles which, as a rule, are more active than the peripheral acini.

The females exposed to cold had significantly heavier thyroids than laboratory controls of the same sex. The mean weight of the thyroid gland of the 13 experimental females was 34.8 mg., while the corresponding average for the 13 controls was 23.6 mg., the increase in weight in this case amounting to 47 per cent (Table 2). Again, however, this increase in weight could not be ascribed to heightened secretory or excretory activity of the glands, in so far as it could be judged from histological appearance. The same "resting" and "storing" glands as were observed in the thyroid of the male were likewise encountered here. However, the tendency shown by the thyroid of some experimental males toward an increase in number of small follicles was even more pronounced in the cold-room females and may well account for their heavier thyroids.

The histological structure of the thyroid of the female did not differ from that of the male. The "resting" follicle displayed the same squamous epithelium, compressed nuclei, and abundance of stored dense colloid. The "storing" follicle was characterized

[4] In every case both thyroids were weighed fresh.

by a similar cuboidal epithelium, by colloid floccules or globules in the apical portion of the epithelial cell, and by a usually thinner colloid. In two experimental females the thyroid colloid was quite thin; it stained a faint blue with Mallory and contained fine droplets of still lighter material. Nevertheless, this type of colloid was found also in the thyroid of one control female, so that it cannot be considered a result of the treatment.

It would seem, therefore, that neither the lower environmental temperature nor the prolongation of the high state of sexual development resulting from extended exposure to lower environmental temperature had any visible effect upon the thyroid gland of the male or female *C. tridecemlineatus*. Nor did exposure of the animal to ultra-violet radiation appear to influence visibly the thyroid gland of either sex.

Prolonged and continuous exposure of the male ground squirrel to a temperature of +4° C. failed to affect the weight of the adrenal gland. The average weight of the adrenal in the experimental series is 25.9 mg. and in the control lot 27.6 mg. (Table 1). Histologically, however, definite effects of the treatment were demonstrable.

In a study of the seasonal changes taking place in the adrenal gland of the same species, conducted by the senior writer (1934), it was shown that in the male the outer reticular subzone of the adrenal cortex (noncommittally termed by the writer "reticularis A") becomes particularly wide, well developed, and conspicuous during the peak of reproductive activity, i.e., during April and May. The cells of this subzone are characterized by large size, high cytoplasm-nucleus ratio, and by a deeply staining area surrounding the nucleus and usually leaving a narrow, light perinuclear ring and a similar peripheral ring. The reticularis A cell is apparently a highly specialized cell which has gradually developed from a cell of the fasciculate zone. Its physiological significance has not been determined.

Concomitantly with this widening and accentuation of cellular characteristics of the reticularis A during the period of high sexual activity, there is a general expansion of the reticular zone, partly at the expense of the fasciculata, as well as an expansion of the cortex in general. The cortex-medulla ratio as calculated by the planimetric method (Zalesky, 1934) is highest during the period of breeding capacity. The weight of the gland is also highest during this phase of the annual reproductive cycle.

Similar tendencies are displayed by the male ground squirrel subjected to the cold-room treatment. The reticularis A, on the whole, shows greater development, both qualitatively and quantitatively, in the adrenals of the experimental animals. The adrenals of some controls sacrificed during November and December lack a reticularis A altogether. This expansion of the outer reticularis in the adrenals of the experimental animals occurs at the expense of the fasciculate layer. The weight of the gland as a whole does not increase. Nor is the cortex-medulla ratio consistently altered. In one adult experimental male it amounted to 5.9:1 and in another to 9.8:1, as compared to 5.7:1 in the standard adult control.

The average width of the reticularis A in the control animals was slightly over 3 cells, whereas in the experimental animals it was over 8 cells.

In the female these changes were even more pronounced than in the male. This is equally true of the normal seasonal cycle in the adrenal gland of the female. Whereas in late autumn and winter, i.e., during the period of anoestrum, there is usually no trace of reticularis A in the adrenal cortex, in April and May, i.e., during oestrum and pregnancy, this subzone may reach 12–15 cells in width and appear as the most con-

spicuous layer in the adrenal cortex. Similarly, the range of seasonal variations in the weight of the gland is considerably wider in the female than in the male. Thus, during the period of sexual inactivity the mean weight of both adrenals of the female is 12.2 mg., while at oestrum it amounts to 22.3 mg., and during pregnancy to 31.0 mg.

The cortex-medulla ratio also shows, in the spring, an increase to 10.9:1 from the November low of 5.2:1.

Similar differences were observed in the present investigation between the control females and the females whose high degree of sexual development has been prolonged by exposure to an environmental temperature of +4° C. The mean weight of the adrenal gland of the 13 control females was 15.8 mg.; the corresponding average for the 13 experimental females amounted to 24.9 mg., representing an increase of 58 per cent (Table 2). The cortex-medulla ratio in a typical experimental female was found to be 9.6:1; whereas the adrenals of all controls showed a virtual absence of the reticularis A subzone, in the adrenals of the experimental animals this subzone was 4–8 cells wide. The cells of the fasciculate zone were likewise larger and more highly developed in the adrenals of the experimental females than in those of the controls of the same sex.

In either male or female the zona glomerulosa of the adrenal cortex, as well as the adrenal medulla, did not seem to be visibly affected by the cold-room treatment.

Similarly, no observable gross or microscopic effects were produced in the adrenal of either the male or the female by exposure of the animal to ultra-violet radiation.

## DISCUSSION

The present investigation has shown that exposure of either males or females of the hibernating rodent *C. tridecemlineatus* to extended and continuous low environmental temperatures failed to maintain the thyroid gland in a state of hyperactivity, in spite of the fact that it prevented sexual retrogression. Although at the time of autopsy the reproductive organs of the experimental animals exhibited a high degree of anatomical development, the thyroids presented evidence of low activity characteristic of the thyroid of the ground squirrel in hibernation. Apparently, the well-developed, potentially functional gonads failed to influence the thyroid when the environmental conditions had an inhibiting effect upon thyroid activity.

In the rat exposure to cold has been reported to have the opposite effect. It caused an outburst of heightened activity in the thyroid gland. The epithelium increased in height, and the colloid became liquefied and tended to disappear from the intrafollicular lumina, apparently being resorbed into the blood stream (Kenyon, 1933; Fischborn, 1935; Baillif, 1937). We must, however, bear in mind that in the case of the ground squirrel we deal with an animal upon which, by a special evolutionary adaptation, lower environmental temperatures have a physiologically inhibitory influence.

These results are compatible with the writers' previous conclusions regarding the thyroid-gonad interrelationships in the ground squirrel under investigation. The senior writer (1935) in a seasonal study of the *Citellus* thyroid has shown that, whereas heightened activity is generally observed in the thyroid gland of both the male and the female during the breeding season, this hyperactive phase in the annual thyroid cycle, characterized by high follicular epithelium and increased colloid absorption, persists throughout the summer and early autumn, i.e., throughout the animal's nonhibernating period. Therefore, it is not necessarily a result of gonadal influence but may be a phenomenon of metabolic origin. Furthermore, evidence was then presented to the effect that either

administration of male or female sex hormones or castration failed in both sexes to affect the thyroid gland, either grossly or microscopically.

In the adrenal gland, prolongation of high sexual development by means of low environmental temperature tends to stimulate high cortical development and differentiation of the reticular zone. This observation is in complete accord with results of a previous study (Zalesky, 1934), which show that a similar adrenal hypertrophy and differentiation occur in the normal reproductive cycle of both sexes during the period of breeding capacity, as well as following administration of gonadal stimulants or homologous sex hormones.

Thus we see that certain physiological processes in the adrenal cortex of the hibernating mammal under investigation are decidedly correlated with the animal's reproductive activities. The anatomical evidence of these processes has been described. Their significance, however, in the endocrine complex underlying reproductive activities will not have been determined until several additional phases of the adrenal-sex relationship have been cleared up. In that direction our investigation is now being further pressed.

## SUMMARY

Twenty-four male and 13 female ground squirrels, *C. tridecemlineatus*, were individually caged at a temperature of +4° C. Twenty-five males and 13 females, captured in the field at the same time as the experimental animals, were kept at room temperature as controls. The experiments were begun in April and early May, i.e., during the period of breeding activity and high sexual development. All animals in the cold room maintained their high sexual development, while all controls, on the other hand, regressed sexually within several months to a practically infantile condition.

Neither the low environmental temperature nor the prolongation of the high sexual development under the influence of low temperature seemed to affect, in either sex, the degree of physiological activity of the thyroid follicle as judged from its histological appearance. At autopsy in the autumn and winter the experimental animals, as well as the controls, yielded thyroids of the "resting" or "storing" type with little or no colloid resorption, low or cuboidal follicular epithelium, and abundant, usually dense colloid.

The adrenal gland of the experimental animals showed, at autopsy, a more highly developed and differentiated cortex than that of the control animals and approximated the cell picture normally seen in the adrenal cortex of the ground squirrel during the breeding period.

The adrenal medulla did not appear to be visibly affected in either sex by the cold-room treatment.

Exposure for 4 hours daily to ultra-violet radiation in addition to the cold-room treatment apparently did not affect the thyroid or adrenal glands.

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# GROWTH OF PROTOZOAN CULTURES. II. LEUCOPHRYS PATULA AND GLAUCOMA PYRIFORMIS IN A BACTERIA-FREE MEDIUM

(Six figures)

MORDEN G. BROWN[1]
School of Biological Sciences, Stanford University, and Marine Biological Laboratory, Woods Hole

THE growth of a population of organisms is usually influenced by so many different environmental and other factors that true analysis of the resultant population changes is impossible, and one can do little more than fit empirical curves to the data. The purpose of this study is to investigate a simple population in a limited environment and to attempt in this special case so to control the conditions of experimentation that the interaction of factors responsible for rate of growth can be determined. For this work protozoan populations seem most suitable, but even the majority of these present great difficulties in control, since the food is bacteria. Some protozoa, though, can be grown in a bacteria-free medium and grown through a sufficient range of densities so that the phases of growth are distinctly separated. Results of some studies on such a population are reported in this paper.

## THE PROTOZOA AND THEIR CULTURE

*Leucophrys patula* and *Glaucoma pyriformis*[2] are rather closely related holotrichous ciliates of the family Frontoniidae. The cultures of these organisms used in this growth study are from California, the *Leucophrys* (*L*) isolated in 1934 by W. H. Furgason from near Stanford University and the *Glaucoma* (*G*) in 1930 by J. O. Thomas from near Monterey. Both organisms were freed of all bacteria by individual washing (modified Parpart [1928] technique) and migration through dilute agar (0.5 per cent or less agar-agar). The *G* were then cultured in 0.2 per cent Difco yeast extract, and the *L* on the *G* culture. Preliminary tests showed that *Colpoda duodenaria* was also suitable as food for the *L* and that *Chilomonas paramecium* was unsuitable. Though culture of the very closely related *Colpoda steinii* under sterile conditions has been described by Cehler (1919), no successful culture medium was found in experiments on bacteria-free culture of *C. duodenaria*, and hence this protozoön was not used for further experiments. The *Colpoda* cultures for these preliminary tests were kindly given to me by A. G. R. Strickland, and the attempts at bacteria-free culture of *Colpoda* were also made by Strickland.

[1] Now at Washington University, St. Louis.

[2] This organism is the same as the *Colpidium campylum* used by Taylor, Thomas, and Brown (1933), as the *Glaucoma pyriformis* used by Phelps (1935 and 1936), and as the *Tetrahymena glaucomaformia* described by Furgason (1936) and used by Giese (1938). A paper describing this organism in detail is to be published by Furgason (Arch. f. Protistenk., in press).

## CULTURE CHAMBER AND COUNTING TECHNIQUE

Experimental cultures were grown in half-filled, 1-liter Florence flasks plugged with cotton. Inoculation and sampling were achieved through use of a U-shaped, 5-mm. glass tube and test tubes to which 4-mm. arms, plugged with cotton, have been attached as shown in Figure 1. The short test tubes were used for inoculation, and the longer ones for removal of samples. Control of the inoculation and sampling was by means of a rubber tube from the side arm to the operator's mouth. Care was always taken before withdrawing samples to mix the organisms thoroughly by shaking the flask and blowing back the liquid that was in the lower part of the 5-mm. tube.

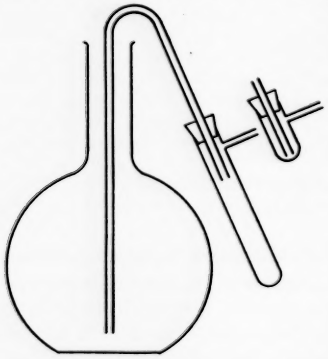

FIG. 1.—Culture flask for study of protozoan populations.

After removal of about 2 ml. to the test tube and replacement of this tube by another similar, sterile one, samples were removed from the test tube with calibrated pipettes and placed in series of small drops on a glass plate. It was found advisable in using the micropipette always to empty it completely, since the last drop contains an excessively large number of organisms, indicating that some of the protozoa probably adhere to the glass until stripped off by the surface film.[3] The number of organisms of each species in each drop on the glass plate was then counted; if there were too many protozoa in a drop for an accurate count of the swimming organisms, swimming was stopped by blowing a puff of air containing acetic acid fumes onto the drop, and then the killed organisms were recounted at a higher magnification.

## EXPERIMENTAL DATA AND ITS ANALYSIS

A test run of growth of *G* alone at 25° C., using the above technique, is shown in Figure 2; the curve is similar to those obtained by Phelps (1935 and 1936). In this experiment the growth-rate in the exponential phase is 6.1 generations per day as compared with 5.6 generations per day reported by Phelps (1935), 6.3 generations per day reported by Phelps (1936), and 6.36 generations per day reported by Hetherington (1936), all tests made with Difco yeast extract as the culture medium and at a temperature of 25° C.

In all plots of growth curves in this report the theoretical curves for unlimited growth are represented by continuous lines, and the deviations due to limitations within the organism (lag phase of adjustment to the environment) and imposed upon the organism by the environment (phase of negative acceleration of growth) have been shown by broken lines drawn freehand.

The theoretical curves for the double culture are determined by the conditions that the organisms divide to produce two daughters at regular intervals and that the rate of

[3] Phelps (1935) had found that the *G* would stick to the wall of a capillary tube through which a culture was flowing; in his 1936 work he eliminated any error due to organisms sticking to his pipettes by killing the *G* before pipetting and counting. Hetherington (1936) also uses a technique designed to overcome errors caused by adherence of *G* to the walls of his pipettes.

capture of $G$ by $L$ is constant. Under these conditions the equations for rates of growth are

$$\frac{dN_L}{dt} = r_L \cdot ln2 \cdot N_G \tag{1}$$

and

$$\frac{dN_G}{dt} = r_G \cdot ln2 \cdot N_G - r_c N_L , \tag{2}$$

where $r_L$ and $r_G$ are the division rates for $L$ and $G$ in units of generations per unit time and $r_c$ is the rate of capture of $G$ by $L$ in units of $G$ captured per unit time per $L$. Upon

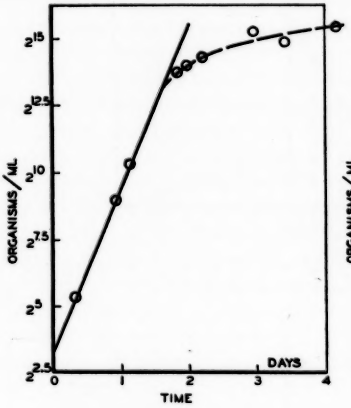


FIG. 2

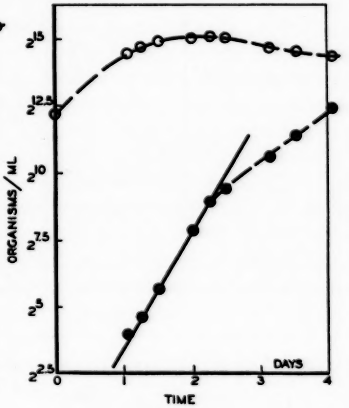


FIG. 3

FIG. 2.—Growth of *G. pyriformis* at 25° C. in 0.2 per cent Difco yeast extract

FIG. 3.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract

integration and introduction of the initial conditions that when $t = 0$, $N_L = (N_L)_0$ and $N_G = (N_G)_0$, equations (1) and (2) become

$$N_L = (N_L)_0 2^{r_L t} , \tag{3}$$

and

$$N_G = (N_G)_0 2^{r_G t} - \frac{r_c (N_L)_0}{(r_G - r_L) ln2} (2^{r_G t} - 2^{r_L t}) . \tag{4}$$

In calculating the theoretical curve for $N_G$, the value of $r_G = 6.1$ generations per day from the data of Figure 2 has been used. The values of $r_L$ and $(N_L)_0$ have been taken directly from the separate graphs of the growth of the $L$ alone, then the experimental values for $N_G$ and $N_L$ at two times were introduced into equation (4) and the resulting simultaneous equations then solved for values of $(N_G)_0$ and $r_c$. For example, in the experiment plotted as Figure 4 the values of $r_L = 4.0$ generations per day and $(N_L)_0 = 14.9$ were taken from the data on growth of $L$ alone, then the experimental data

$$\left\{ \begin{array}{l} t = 0.833 \text{ days} \\ N_L = 150 \\ N_G = 11{,}400 \end{array} \right\} \quad \text{and} \quad \left\{ \begin{array}{l} t = 1.416 \text{ days} \\ N_L = 740 \\ N_G = 3{,}800 \end{array} \right\}$$

were introduced into equation (4), and values of $(N_G)_0 = 1{,}710$ and $r_c = 190\,G$ captured per day per $L$ were obtained. Points on the theoretical curve were then calculated, and it was found that the curve fitted the experimental data for $N_G$ at $t = 0$ and $t = 0.2085$

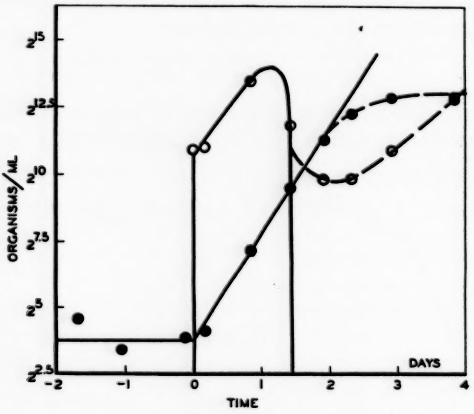


FIG. 4.—Growth of *L. patula*, ●, on culture of *G. pyriformis*, ○, at 25° C. Food organisms were absent in the culture flask until the *Glaucoma* were introduced at zero time.

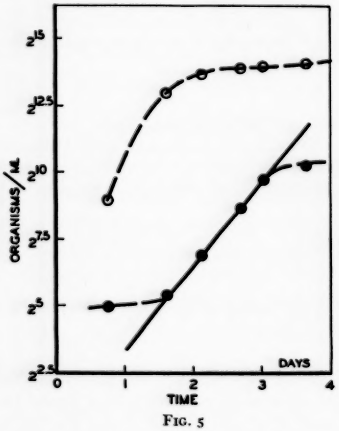


FIG. 5

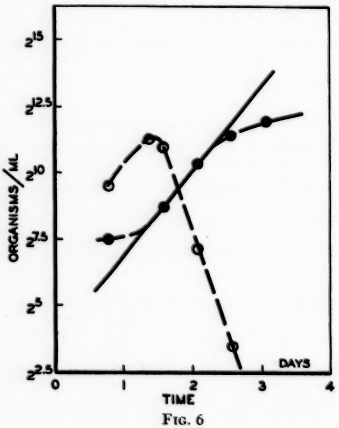


FIG. 6

FIG. 5.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract
FIG. 6.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract

days. Unfortunately, in most cases the conditions were such that the period throughout which both organisms were growing exponentially was too short for good determination of $r_c$ by this method. The value of $r_c$ in units of $G$ captured per $L$ between each fission is $r_c/r_L = 47$.

The mean value of $r_L$ is 3.7 generations per day (Figs. 3, 4, 5, 6, and two experiments not shown by graphs). The values averaged for $r_L$, however, come from separate series of experiments in which the conditions were apparently not identical: values of 4.3, 4.2, and 4.0 were found in one series of experiments (e.g., Figs. 3 and 4); and values of 3.4, 3.3, and 3.0 were found in a later series (e.g., Figs. 5 and 6). Though there was no lag phase in any case in the first series and one present in each case in the second series, there is not thought to be any correlation between a lag phase and a lower fission rate in the subsequent exponential phase of growth.

## DISCUSSION AND SOME QUALITATIVE OBSERVATIONS

Any representation of growth in the lag phase or the phase of negative acceleration of growth by theoretical curves seems quite inadvisable at present, and, indeed, for the phase of negative acceleration of growth it seems doubtful that the same formulation will fit cases in which different factors are limiting. However, these bacteria-free protozoan cultures seem very suitable for the experimental separation of factors involved in these phases, and through further experimentation it should be possible to examine the relations of environmental factors to phases of nonexponential growth and to give a sound basis for any exact mathematical formulation of growth in these phases.

Though quantitative analysis of phases other than those of exponential growth is not attempted, a number of qualitative observations should be noted. In the experiments shown as Figures 3 and 5 termination of exponential growth is apparently the result of factors due to high concentration of organisms, whereas in the experiments shown as Figures 4 and 6 termination of exponential growth is apparently caused by depletion of food organisms.

In Figures 3 and 5 it is seen that, though the concentration of *G* is such that they are in the phase of negative acceleration of growth, exponential growth of *L* is still found. One might suppose then that the *L* are less sensitive to low oxygen tensions than the *G*, since Phelps's studies seem to show that the limiting factor for growth of *G* under conditions similar to these is oxygen tension. However, since in several instances the *G* have been found to multiply after the *L* have entered a phase of negative acceleration of growth and since the *L* are larger than the *G*, it seems advisable to await direct measurement of growth under controlled conditions of oxygen tension before attempting an explanation of the nature of the intermediate factors terminating the exponential growth phase in these cultures in which concentration of organisms is a limiting factor.

In Figures 4 and 6 it is seen that exponential growth of *L* continues for a time after the rate of capture of *G* by *L* ($r_c$) is no longer constant but has decreased. This decrease in $r_c$ is shown by the values of $N_G$ lying above the extrapolation of the theoretical curves. The delay in decrease in $r_L$ after $r_c$ has decreased is most probably due to the presence of considerable food reserves in the undigested food vacuoles and in the protoplasm of the *L* which permit the continuation of regular fission without capture of many *G*.

It has also been observed that *L* introduced into fresh cultures show a lag phase, e.g., Figures 5 and 6, if taken from the phase of negative acceleration in cultures in which high concentration of organisms has become a limiting factor,[4] but that there is no appreciable

[4] Though the relation between duration of lag phase and position in the phase of negative acceleration of growth from which the organisms were taken has not been worked out, it was observed that organisms need not progress far into the phase of negative acceleration of growth before showing a definite lag phase, e.g., organisms taken from the experiment shown as Figure 4 at 2.2 days showed a very pronounced lag

lag phase if *L* are taken from the phase of negative acceleration or stationary state in cultures in which depletion of food organisms is the limiting factor, e.g., Figure 4, where, prior to zero time, growth is limited by absence of food organisms and where, upon introduction of *G*, the potential growth-rate for the environment is realized after little or no lag phase.

Further study of growth of *L* in the absence of food organisms showed that the *L* would maintain themselves for many days in the sterile Difco yeast-extract solution alone and even increase in number to a slight extent. It was noted that addition of yeast powder such as used by Hetherington (1936) improved growth in the absence of food organisms, and it has since been found by J. O. Thomas (personal communication) that the *L* will grow fairly well on the yeast autolysate used by Phelps (1936), without introducing any other organisms. Thus in these growth experiments the 47 *G* captured between each fission of *L* doubtless does not represent all the food obtained in this interval, since the experiments were carried out in a nutrient solution.

## SUMMARY

1. Methods for culture and growth measurement are described for the double culture of *L. patula* and *G. pyriformis* in a bacteria-free medium.

2. Growth of *L. patula* is found to be exponential at a rate of 3.7 generations per day at 25° C. up to concentrations of the order of 2,000 per milliliter under the conditions of the experiments.

3. Analysis of the growth curves of *Glaucoma* and *Leucophrys* together to obtain the rate of capture of *Glaucoma* by *Leucophrys* is shown. It is found that approximately 50 *Glaucoma* are captured between each fission of *Leucophrys*.

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phase. This subsequent lag phase of organisms taken at 2.2 days in this experiment indicates that in this case concentration is a limiting factor, though both concentration and depletion of food are probably factors in this particular instance. That concentration was a limiting factor might also have been inferred from the later growth of *G* as seen in the figure, for in experiments such as that of Figure 6 in which low concentration of G becomes a limiting factor at a lower concentration of *L*, no *G* were found at later periods.

# BIOCHEMICAL ASPECTS OF THE DIFFERENTIATION OF THE FEMALE HONEYBEE (APIS MELLIFERA L.)[1]

(Seven figures)

R. M. MELAMPY, E. R. WILLIS,[2] AND S. E. MCGREGOR
U.S. Department of Agriculture, Bureau of Entomology and Plant Quarantine, and Department of Zoölogy, Louisiana State University

ALTHOUGH the dimorphism existing in the female honeybee (*Apis mellifera* L.) has claimed the attention of naturalists and biologists for centuries, little attempt has been made to study the physiology of the development of the two castes. The queens and workers are produced from identical larvae, as it is possible to rear queens from any female larvae by providing them with the proper food and care during early life. Furthermore, queens require between 12 and 13 days to complete their larval and pupal development after hatching from the egg whereas the workers complete their development in about 18 days. According to Snodgrass (1925), for the first two days after hatching all female larvae apparently receive the same diet. This diet is royal jelly, a secretion of the pharyngeal glands of the workers. During the third day the diet of the larvae that are to become workers is altered, but the queen caste continues to receive royal jelly. This difference appears to be a factor in bringing about the morphological and functional differences between the two female castes.

Melampy and Jones (1939) have shown that royal jelly from queen cells containing larvae between 3 and 4 days old has the following approximate chemical composition: moisture 66 per cent and dry matter 34 per cent, the latter consisting of protein 36 per cent, fat 16 per cent, total reducing substance (calculated as glucose) 37 per cent, ash 2.4 per cent, and undetermined material 8.6 per cent. Royal jelly is a good source of vitamin $B_1$, containing 1.0–1.5 I.U. per gram, but it contains no demonstrable amount of vitamins A or C.

The physiology of caste production in social insects is not clearly understood; however, several investigators have offered suggestions as to possible mechanisms involved. Huxley (1932, p. 62) reported that the workers and soldiers of the ants *Anomma nigricans* and *Camponotus gigas* represent a series of size forms of a single genetic type and "that the differences in size can only be supposed to be brought about by definite treatment of the larvae by their nurses, the largest types being fed to the limit, the smallest types being deprived of food, and so forced to pupate, while still quite small larvae." Castle (1934) has demonstrated that feeding alochol and ether extracts of functional queens of the termite *Termopsis angusticollis* Hagen to isolated groups of fifth and sixth instars retards the development of female supplementary reproductives.

Hill and Burdett (1932) reported experiments from which they concluded that "the bees add vitamin E to royal jelly, on which queens are raised, and withhold it from

[1] The writers are indebted to Professor A. E. Emerson, of the University of Chicago, and Dr. M. Somogyi, of the Jewish Hospital, St. Louis, Missouri, for their helpful suggestions.

[2] Graduate Fellow, Louisiana State University.

worker larval food." Mason and Melampy (1936) assayed this secretion and found vitamin E to be absent, and their finding was confirmed by Schoorl (1936), by Evans, Emerson, and Eckert (1937), and by Haydak and Palmer (1938). Heyl (1939) has suggested the presence of a gonadotropic hormone in royal jelly because the injection of dilute sodium hydroxide and aqueous pyridine extracts of the secretion caused precocious development of the Graafian follicles in immature rats. However, this finding needs verification. In a recent review Emerson (1939) has suggested that the secretions and exudates consumed by social insects may contain specific substances (coenzymes) "which, when activated, can stimulate or inhibit development and behavior."

The work reported herein deals with the chemical changes associated with the development and differentiation of the two female castes, being part of a study of the physiology of reproduction in the honeybee. This dimorphism due to differences in nurture of individuals having the same gene complex furnishes an excellent example of the influence of environment on insect development.

## MORPHOLOGICAL DIFFERENTIATION BETWEEN QUEEN AND WORKER

The queen and worker castes of the honeybee are adaptive to their specific function as imagoes and are not degenerate, each having many specialized characters. The queen is characterized by a short proboscis, notched mandibles, small brain, long sting, rudimentary pharyngeal glands, highly developed functional ovaries, and long life-span. On the other hand, the worker has a long proboscis, specialized mandibles, large brain, short sting, functional pharyngeal glands, undeveloped ovaries, and a comparatively short life-span. The primary function of the queen is reproduction, whereas the workers have more varied duties, such as collecting and storing nectar and pollen, secreting wax and building comb, providing protection, and rearing brood.

The mature queen honeybee weighs about 200–225 mg.; the writers obtained an average weight of 216.99 $\pm$ 1.63 mg. for 100 laying queens. The average weight of 2,425 workers they found to be 118.90 $\pm$ 0.26 mg.

The reproductive organs of the queen are highly developed and occupy a large part of the abdominal cavity. Eckert (1937) reported an average of 340 ovarioles in the ovary. Nelson (1924) noted that the rudiments of ovaries are much larger in the queen larvae than in the worker larvae, an indication that their development is greatly accelerated during later larval life. He also found their structure to be similar in the two castes except that in the queen larvae the presumptive rudiments of the ovarian tubules are both longer and more numerous. Oertel (1930) observed that in the worker larvae the number of ovarian tubules in each ovary decreases to 12–15 approximately 30 hours after sealing and that only 3 or 4 remain 120 hours after sealing. No further changes were observed in the workers 240 hours after sealing. Hambleton (1928) found a rudimentary bursa copulatrix, spermatheca, and spermathecal gland connected with the vagina in the adult worker honeybee. Mature eggs are sometimes produced by workers.

## EXPERIMENTAL PROCEDURE

### REARING AND PREPARATION OF INSECTS

The method for rearing the honeybees used in this work has been described by Melampy and Willis (1939). The larvae were removed from the queen cells or brood frames for analysis, washed with tap water, rinsed with distilled water, and placed between

sheets of filter paper to remove excess water. The guts were not removed since it is difficult to remove completely the alimentary tract containing the undigested food of the immature honeybee. There is no connection between the mid-gut and the hind-gut until 5–10 hours after the larva is sealed, according to Oertel (1930). At this time the waste material in the mid-gut passes to the hind-gut, and 20 hours after sealing it is entirely evacuated. Since the pupae and newly emerged adults were free from adhering material, it was not necessary to wash them.

### ANALYTICAL METHODS

Growth, moisture, dry matter, nitrogen, and total lipid were determined at 24-hour intervals, whereas the total reducing substances, calorific value, and ash content were determined at 3-day intervals. Data from chemical analyses used in constructing Figures 2–7 represent averages of 4–10 determinations made over a period of 6 months. The amount of insect tissue used for the various chemical determinations varied with the stage of development of larvae and pupae.

*Growth.*—The growth data were obtained by weighing representative lots of insects at 24-hour intervals during the life-cycle. The insects were weighed in groups of 15–200 individuals, depending upon the stage of development. The average values used in plotting the growth curves represent the average weights of at least 200 individuals and not more than 1,200.

*Moisture, total nitrogen, and ash.*—The moisture determinations were made in a vacuum oven at 60° C. The total nitrogen was determined by the standard Kjeldahl procedure and represents tissue and chitin nitrogen as well as end products of nitrogen catabolism such as uric acid. Ash determinations were made in a muffle furnace at about 600° C.

*Total lipid.*—The total lipid was determined by the Kumagawa and Suto procedure as used by Slifer (1930). Two or three grams of living insects was dried *in vacuo* at 60° C. The dried tissue was pulverized and transferred to a tall beaker for saponification. A mixture of 25 cc. of 25 per cent sodium hydroxide and 5–10 cc. of ethyl alcohol was added, the beaker covered with a watch glass, and the whole heated for 3 hours on an electric plate. It was necessary to maintain a low temperature and to stir the mixture occasionally. The reaction was allowed to continue overnight at room temperature. The soap solution was then transferred quantitatively to a glass-stoppered bottle, and the fatty acids were liberated by adding 25 cc. of concentrated hydrochloric acid. After the mixture had cooled to room temperature, 40 cc. of pure ethyl ether was added. The bottle was shaken thoroughly and allowed to stand until the ether and aqueous layers had separated, when the ether layer, containing the lipids, was drawn off into an Erlenmeyer flask. The extraction was repeated six times, 25-cc. portions of ether being used, and the combined extracts were then evaporated at 35° C. The lipid residue was taken up in purified petroleum ether. The petroleum-ether extract and the subsequent petroleum-ether washings were filtered through fat-free filter paper into weighed small beakers, and the ether was allowed to evaporate at room temperature. The fat in the beakers was dried for 1 hour at 60° C. and cooled in a desiccator before being weighed. The total lipid represents the fatty acids as well as such ether-soluble, nonsaponifiable substances as the sterols.

*Total reducing substances.*—The total carbohydrate content of the tissue was deter-

mined in terms of total reducing substances calculated as glucose. It is believed that the amount of total reducing substances is an index of the carbohydrate content, because after fermentation with washed yeast the filtrates produced little, if any, color with the sugar reagent. This test demonstrates the absence of interfering substances, according to Somogyi (1928).

The macerated tissue was placed in a 50-cc. centrifuge tube, and 10 cc. of 1.2 N sulphuric acid added for each gram of tissue. The tube was closed with a rubber stopper, which was provided with a 2-foot glass tube serving as a reflux condenser. The tubes and their contents were heated for 3 hours in a boiling-water bath. When the digests were cool, they were neutralized with a saturated solution of barium hydroxide, phenolphthalein being used as an indicator, diluted to 50 cc., and filtered. The amount of total reducing substances was determined in the filtrate by the dinitrosalicylic acid method of Sumner (1924).

*Calorific value.*—The calorific value of the moisture-free tissue of queens and workers was determined at various stages of the life-cycle in order that energy changes accompanying the development of the two castes might be followed. An adiabatic calorimeter was used for this purpose, and the procedure was that described by Long (1934).

## RESULTS

The results of these analyses on queen and worker honeybees are summarized in Table 1 and presented graphically in Figures 1–7.

TABLE 1

AVERAGE WEIGHT, CHEMICAL COMPOSITION, AND CALORIFIC VALUE OF THE DEVELOPING WORKER AND QUEEN HONEYBEE

| Age (Days) | Live Weight (Mg.) | Water (Mg.) | Dry Weight (Mg.) | Nitrogen (Mg.) | Lipid (Mg.) | Reducing Substances (Calculated as Glucose) (Mg.) | Ash (Mg.) | Calorific Value (Cal.) |
|---|---|---|---|---|---|---|---|---|
| | Worker | | | | | | | |
| 3–4 | 25.38 | 21.07 | 4.31 | 0.54 | 0.57 | 0.77 | 0.27 | 23 |
| 6–7 | 136.89 | 103.78 | 33.11 | 2.09 | 5.37 | 12.07 | 1.00 | 177 |
| 9–10 | 130.65 | 102.36 | 28.29 | 1.92 | 5.04 | 8.51 | 1.03 | 161 |
| 12–13 | 131.31 | 105.03 | 26.28 | 2.11 | 4.62 | 8.27 | 1.01 | 145 |
| 15–16 | 126.12 | 105.45 | 20.67 | 2.03 | 2.84 | 3.39 | 0.99 | 110 |
| 18–19 | 113.36 | 92.94 | 20.42 | 2.18 | 1.44 | 0.88 | 1.04 | 104 |
| | Queen | | | | | | | |
| 3–4 | 44.93 | 36.58 | 8.35 | 0.73 | 1.43 | 0.99 | ..... | 51 |
| 6–7 | 262.34 | 197.62 | 64.72 | 5.72 | 12.84 | 13.17 | 0.21 | 425 |
| 9–10 | 242.05 | 189.09 | 52.96 | 5.10 | 10.44 | 9.03 | 0.24 | 318 |
| 12–13 | 195.54 | 155.47 | 40.07 | 4.96 | 3.91 | 3.48 | 0.21 | 214 |

## DISCUSSION OF RESULTS

*Growth.*—The growth curves in Figure 1 are semilogarithmic representations of increase in mass per individual insect plotted against age. They demonstrate that the two castes develop at approximately the same rate during early larval life, after which the growth of the worker is retarded, and the time necessary for development is extended beyond that required by the queen. The weight of a newly hatched larva is approximately 0.1 mg., and the maximum live weight attained by the queen during development is more than 2,500 times greater. On the other hand, the worker increases about 1,400 times. Elser (1929) reported a 7-day-old queen weighing nearly 300 mg.

FIG. 1.—Growth curves for the queen and worker honeybees. *P* indicates the time of pupation

The growth curve for the worker is in agreement with the curve for the same caste reported by Nelson, Sturtevant, and Lineburg (1924).

*Dry matter and moisture.*—The highest percentage of dry matter and the lowest percentage of water occur at about the 5–6-day stage for the worker and the 6–7-day stage for the queen (Fig. 2). However, the absolute amounts of dry matter and water per individual insect (Fig. 3) are both at a maximum at these periods. The queen and worker parallel each other in percentages of dry matter and water, although the former develops at a greater rate.

It is suggested that the increase in dry matter for both castes during the larval stage is due to the formation of tissues, organs, and reserve materials. At this time the anabolic processes predominate over the catabolic; hence the observed increment in dry matter (Figs. 2 and 3, Table 1) is the difference between the amounts synthesized and the amounts utilized.

During the pupal period there is a gradual decrease in the percentage of dry matter for both queens and workers, which may be accounted for by the utilization of reserves, spinning of the cocoon, and evacuation of the gut.

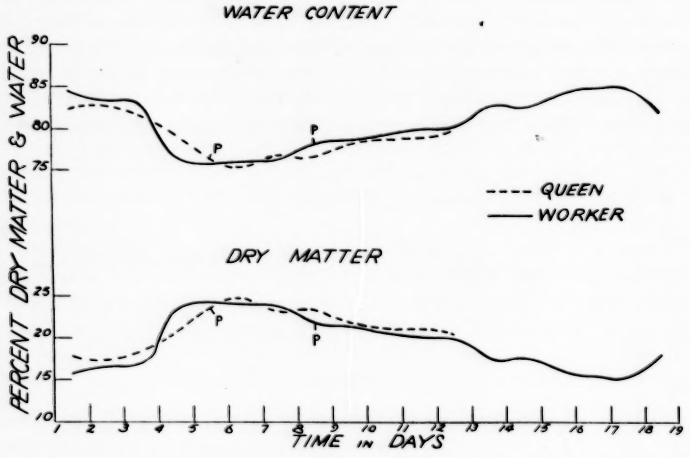


FIG. 2.—Percentages of dry matter and water in the queen and worker honeybees during development. *P* indicates the time of pupation.

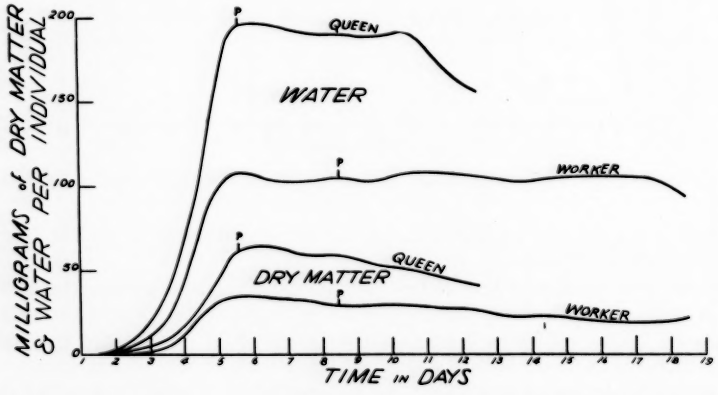


FIG. 3.—Dry matter and water content in milligrams per individual queen and worker honeybees during development. *P* indicates the time of pupation.

The gradual increase in the percentage of water during pupation is a result of a nearly constant amount of water per individual insect and a decreasing total live weight. The decrease in the total weight is due principally to the utilization of energy reserves, which constitute a significant amount of the dry matter at the beginning of pupation.

However, just prior to emergence there is a drop in the water content per individual insect.

Kellner, Sako, and Sawano (1884) found 1,395 gm. of water and 374 gm. of dry matter in 1,000 silkworm (*Bombyx mori* L.) larvae, but only 830 gm. of water and 339 gm. of dry matter in the pupae, demonstrating that the loss in weight during metamorphosis for this species is due largely to a loss of water.

Water is the most abundant constituent of the developing honeybee, comprising from 76 to 85 per cent of the total live weight of the worker and from 75 to 83 per cent of the queen. The water contained within the insect body is either ingested with the

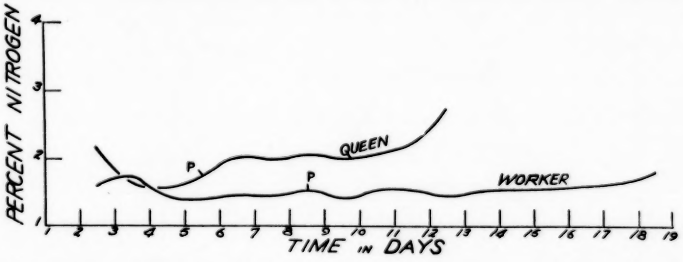


FIG. 4.—Percentages of nitrogen in the queen and worker honeybees during development. *P* indicates the time of pupation.

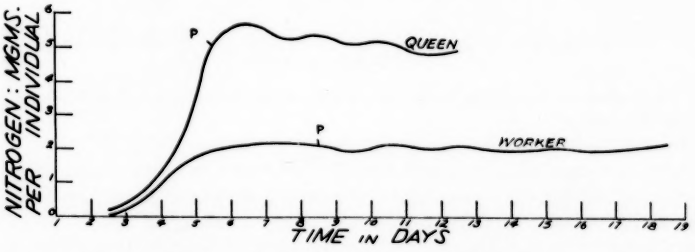


FIG. 5.—Nitrogen content in milligrams per individual queen and worker honeybees during development. *P* indicates the time of pupation.

food or produced within the cells during metabolism. The oxidation of fat and carbohydrate during pupation furnishes energy for adult morphogenesis and simultaneously produces water for cellular functions. The quantity of metabolic water produced by this method depends upon the rate of oxidation and the percentage of hydrogen in the metabolites being consumed. Metabolic water is also produced during growth by dehydrating processes, such as the formation of glycogen from glucose and of structural proteins from amino acids. Metabolic water is of physiological importance to the honeybee during pupation, since no water is ingested during this period.

*Nitrogen content.*—The percentage of nitrogen (Fig. 4) shows a drop between the second and fifth days of larval life, whereas the actual amount of nitrogen (Fig. 5) shows an increase. During this interval total reducing substances (Table 1), total lipid

(Figs. 6 and 7), water content (Fig. 3), and ash (Table 1) are increasing; hence the decrease in the percentage of nitrogen. During the pupal stage there is an increase in the percentage of nitrogen, and this increment is greater for the queen. By this time energy reserves have been depleted, and therefore the nitrogenous constituents are relatively more abundant.

The nitrogenous substances required for histogenesis by the embryonic cells of the imaginal buds are probably furnished by histolysis of the larval structural tissues; hence the quantity of nitrogen remains relatively constant during pupation and adult morphogenesis. Inouye (1912), Evans (1932), and others have reported that the total nitrogen content of other species of insects is constant during pupation.

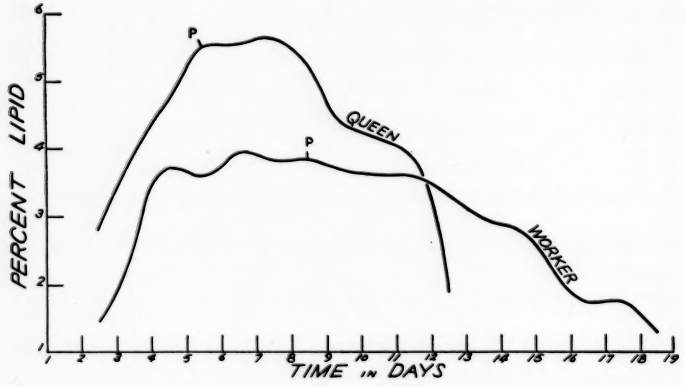


FIG. 6.—Percentages of total lipid in the queen and worker honeybees during development. *P* indicates the time of pupation.

Differences in weight of queens and workers are shown to be due in part to differences in the quantity of nitrogenous constituents. For example, queens 6–7 days old have more than twice as much nitrogen as workers of the same age.

During the larval period there is a marked increase in lipid content which is probably synthesized from dietary fat and carbohydrate. That fatty materials are important in the development of this insect is suggested by their rapid disappearance during metamorphosis (Figs. 6 and 7). This observation is in agreement with the work of Straus (1911). Rudolfs (1926), working with the eastern tent caterpillar (*Malacosoma americana* [F.]), found that the ether-soluble extract increases during the larval period and the first part of metamorphosis and decreases later. Evans (1932) and Yuill and Craig (1937) reported a decrease in the amount of unsaturated fatty acids during the transformation of *Lucilia sericata* (Meig.).

Ditman (1938), working with the corn ear worm (*Heliothis obsoleta* [Fab.]), has shown an increase in the actual fat content and the percentage of fat on a dry-weight basis during the prepupal period. He indicated that there is a conversion of glycogen to fat by the corn ear worm during this period.

*Total reducing substances.*—The data for total reducing substances indicate a storage of carbohydrate during the larval period, which is utilized in pupal development, as

both queens and workers emerge with a small amount of reducing substances. Between the first and second 3-day periods the queens increase their total reducing substances thirteen times and the workers nearly sixteen times. The storage and utilization of total reducing substances parallel lipogenesis and lipid utilization (Figs. 6 and 7).

Ronzoni and Bishop (1929) have shown that the substances produced by the larval honeybee during carbohydrate digestion enter the blood and are partly converted and stored as fat and glycogen and partly remain as free and bound sugar in the blood. Straus (1911) reported that toward the end of the larval period more than 30 per cent of the dry weight is glycogen, whereas Parhon (1909) found that the adults contain 0.14–0.21 per cent of glycogen.

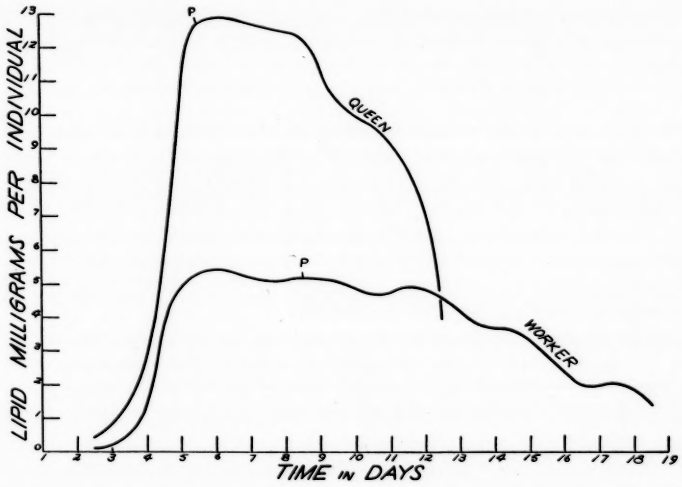


FIG. 7.—Total lipid content in milligrams per individual queen and worker honeybees during development. *P* indicates the time of pupation.

Bataillon (1893), studying the carbohydrate metabolism of the silkworm, found that the quantity of glycogen nearly doubles during the period of spinning and molting but that near the end of pupation nearly all the glycogen reserve has been utilized. Snodgrass (1925, p. 218) suggested that the cells of the fat body function as depots for glycogen reserves. If a mature larva is cut open and placed in iodine, the presence of glycogen in the exposed tissue of the fat body is indicated. Glycogen is found in the liver of higher animals but is also present in the muscles and in other tissues.

Crescitelli and Taylor (1935) found an increasing concentration of reducing substances in the wax moth (*Galleria mellonella* [L.]) during the period of spinning. This finding is in agreement with the results of Bataillon (1893) with the silkworm, Frew (1929) with the blowfly, and Ludwig (1932) with the Japanese beetle (*Popillia japonica* (Newm.). Crescitelli and Taylor demonstrated that during the pupal stage the concentration of reducing substances decreases at first and then rises, and they suggested that, if the reducing substances consist mainly of glucose, a synthesis of this sugar may occur

during metamorphosis of the wax moth. Further citations on carbohydrate metabolism of insects are given by Uvarov (1928).

*Ash content.*—The workers contain more ash than the queens at all ages. The difference in ash content of the food probably accounts for the difference in the amounts of inorganic constituents found in the two castes. Royal jelly contains approximately 0.82 per cent of ash, whereas pollen contains 3 per cent or more.

*Calorific value.*—The calorific value of the tissue increases during the larval period, and there is a considerable expenditure of energy during metamorphosis. The calorific data confirm the changes already presented for dry matter, lipid, and total reducing substances. The reduction in calorific value during pupation is the result of the decrease in the total amount of fat and carbohydrate and, possibly, the accumulation of the end products of protein catabolism. The application of the calorimetric method to insect physiology provides a method of measuring energy utilization by the insect during development.

## SUMMARY

The differentiation of the female castes of the honeybee (*A. mellifera* L.) has been studied from the biochemical standpoint. The findings of this study show that the queens and workers have the same approximate growth-rate during early larval life, and then the growth of the worker caste is retarded, as shown by the nitrogen, total lipid, total reducing substances (calculated as glucose), and calorific value.

This generalization is supported by the following experimental results:

1. The queens attained a maximum live weight of more than 260 mg. during development, as compared with 144 mg. for the workers.

2. The water content comprised 76–85 per cent of the total live weight of the worker and 75–83 per cent of the queen during development. The percentage of water was lowest at about the 5–6-day stage for the worker and 6–7-day stage for the queen. During the pupal period the water content was nearly constant, owing to the production of metabolic water.

3. The queens reached a maximum in nitrogen content at 5.72 mg. per individual, and the workers at 2.18 mg.

4. The highest total lipid content of the queen was 12.84 mg. per individual, whereas that of the worker was 5.37 mg.

5. Total reducing substances reached a maximum of 13.17 mg. for the queen and 12.07 mg. for the worker.

6. The average ash content of the prepupal and pupal stages of the worker was approximately five times that of the queen.

7. The calorific value of the tissue of both castes increased during the larval period and decreased during metamorphosis.

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# PATHS OF WATER EXCHANGE IN THE EARTHWORM[1]

(Eight figures)

A. V. WOLF
Department of Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y.

GEGENBAUR (1853) first suggested the nephridia of the earthworm as excretory in function, enlarging on von Siebold's earlier observation of the expulsion of fluid from the nephridiopores. Overton (1904) noticed in manipulating worms an initial loss of weight which was attributed to loss of nephridial contents. No supporting evidence for the nephridia as the site of origin was found by Adolph (1927) or Maluf (1939), these latter authors holding that most of the weight losses which follow upon successive weighings and handlings of earthworms were from the gut; and it was demonstrated that water might be lost from the mouth as well as from the anus. Darwin (1881) showed that these animals practiced "extra-stomachal" digestion in which they voided fluid from the mouth. Wolf (1938) also observed the voiding of buccal fluid in the beginning phases of dehydration.

It was proposed in the present studies to estimate quantitatively the part played in the water exchanges of the earthworm, *Lumbricus terrestris* L., by both of these paths (gut and nephridia) and by the integument.

## METHODS

Adult worms were stored in moist earth at temperatures ranging from 4° to 13° C. Before use they were transferred to a constant-temperature room at either 18° or 20° C. and allowed to remain in 3 to 6 mm. depth of glass-distilled water in covered glass vessels for at least one night. When being weighed, specimens were lifted from their containers with a blade of a pair of forceps introduced carefully under the body just behind the clitellum. The worm was disturbed as little as possible, drained for a uniform period, and weighed in a tared aluminum cup, after which it was poured back into its container. Worms which were "handled" before weighing were lifted out onto toweling paper, rolled three or four times with dry filter paper, and either weighed directly or else dipped in water so as to become wet, whence they were again removed, drained, and weighed.

Eleven preliminary measurements indicated that, on dipping, water to the extent of 2.68 $\pm$ 0.17 per cent of the body weight adhered to specimens which had been superficially dried by handling. In all cases the sign $\pm$ indicates the standard error.

Three terms should be defined. "Handling" refers to the procedure of rolling a worm with filter paper to dry the surface or to induce the expulsion of fluid from the nephridia and/or gut. "Basal weight" is the weight an individual attained by the expulsion of its water stores such that continued handling induced no further appreciable fluid loss.

[1] The writer is indebted to Professor E. F. Adolph for the interest, encouragement, and constructive criticism he gave to make this work possible and to W. B. Latchford for making the capillary cannulas used.

"Gross weight" (with respect to water) is the weight an individual attained when lying in water for a time long enough that at any succeeding times its water stores did not significantly increase.

## DIURNAL FLUCTUATIONS

Adolph and Adolph (1925) noted that earthworms in tap water maintained constant weight only approximately. Maluf (1939) attributed these "minor fluctuations" to kidney activity. The fluctuations of weight were investigated in the present work by

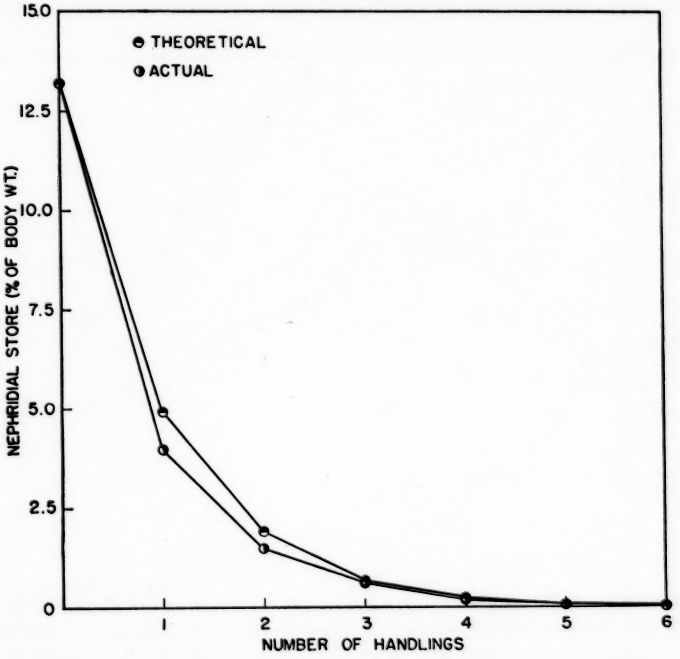


FIG. 1.—The effect of repeated handlings on nephridial water stores. The theoretical curve is plotted from the equation in text. Actual data do not significantly differ from this at any point.

studying the diurnal variations of weight of 12 individuals, each in a separate container of distilled water. Every 24 hours each was weighed to obtain the gross weight, while some of these were then handled to obtain the basal weight. Worms were handled until the difference between two successive weights was less than 1 per cent of the last weight recorded. The interval of time between handlings varied from 2 to 8 minutes; within this range the interval had no significance. The environing water was renewed daily.

Data on 32 worms reduced to basal weight showed that the amount lost per handling, regardless of the initial amount of fluid previously stored, was approximately a constant fraction of the amount in store (Fig. 1). The loss per handling was found to be $63.1 \pm 2.4$

per cent of the amount contained; and this held at least between stores of 23.5 per cent and 2.0 per cent of basal weight, though as basal weight was approached variation increased. From this the suggestion of Adolph (1927) that the loss is a logarithmic function of the number of handlings is confirmed and

$$L_n = L_i (1 - a)^n,$$

where $L_n$ is the fraction of nephridial load after $n$ handlings, $L_i$ is the nephridial load, and $a$ is a constant whose value under these conditions was 0.631 (63.1 per cent).

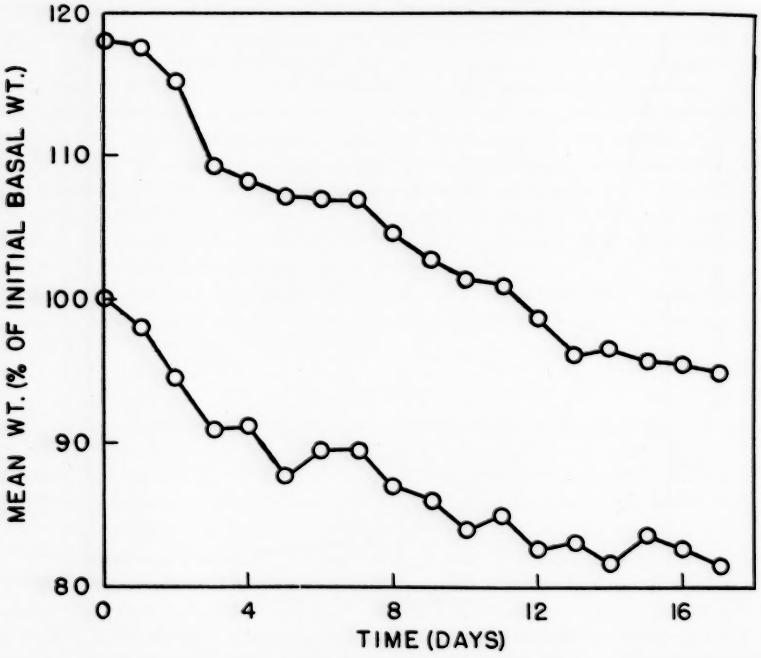


FIG. 2.—Parallel downward trend of gross (upper curve) and basal (lower curve) weights at 17°–19° C. Data are corrected to offset the influence of dying worms on the average weight at a given day. Through the third day, 8 worms are averaged; through the fourth, 7; sixth, 5; twelfth, 4; thirteenth, 3; seventeenth, 2.

Figure 2 shows how gross and basal weights vary with time. The steady fall in weights of worms with time under these conditions is statistically significant. An undetermined but small part of the fall is probably due to defecation in worms whose guts contained some earth and to starvation. It is believed that loss of body fluid through dorsal pores in handling may also be a factor. Permanent loss of salts (accompanied by water) from the worms to distilled water may play a role.

Figure 3 indicates the weight changes of worms placed in a 0.01 M sodium chloride solution. The increase of water load under these conditions is marked. Data were also obtained on a single worm after substituting distilled water for the sodium chloride solution, whereupon loss of body weight followed. These data suggest that loss of salts and hence of water may account for some of the weight loss experienced by worms in distilled water. Maluf (1939) detected losses of chloride from worms only after they were ligated at both ends before their introduction to tap water.

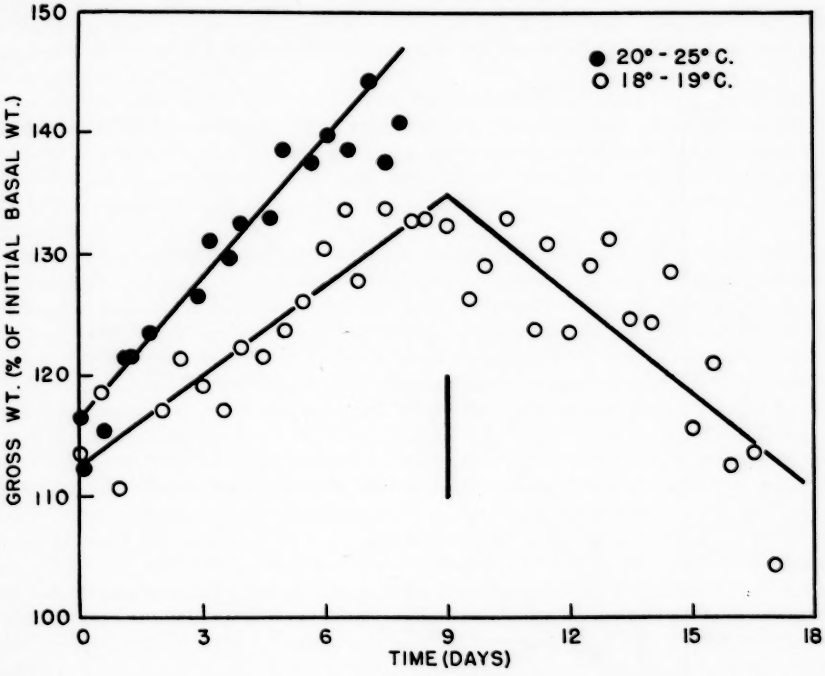


FIG. 3.—Solid circles represent the mean weights of 10 worms in 0.01 M NaCl solution. Open circles represent weight of one worm in 0.01 M NaCl solution until it was transferred on the ninth day to distilled water. The transfer is followed by an abrupt change of slope.

Not only is the fall of basal weight of interest but also the nature of the diurnal fluctuations of the basal and gross weights. The average difference of weight of worms from day to day in percentage of the initial basal weight was found (Fig. 2) to be 2.3 ± 0.3 per cent for the basal weights and 3.2 ± 0.3 per cent for the gross weights of the same worms. The difference is significant. When approximately as many more gross weights of specimens that happened to be available are included in the average difference for gross weights, the result is 3.4 ± 0.2 per cent. These statistics will be discussed in the next sections.

INTESTINAL OUTPUT

From a few data on the recovery of water losses sustained after handling, Adolph (1927) estimated that intake proceeded at a rate of approximately 0.8 per cent of the body weight per hour. Maluf (1939) estimated that a 15 per cent increase in weight

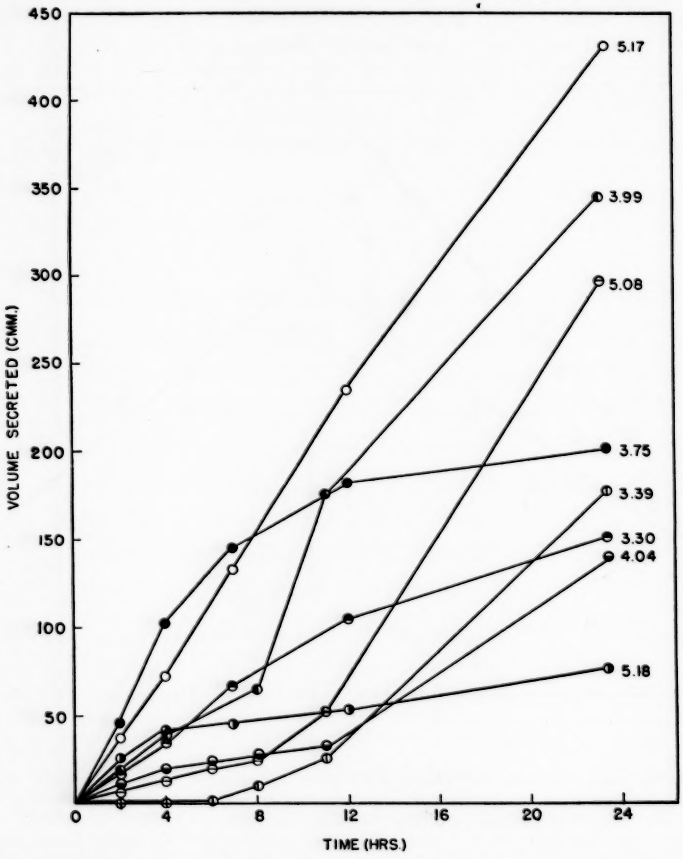


FIG. 4.—Fluid output of the gut. Rates were determined by using slope of curve between last two points. Temperature at zero time was 19°–20° C. but fell off with time to 15°–16° C. Gross weights of specimens are indicated in grams.

could occur in worms transferred from soil to tap water in about 12 hours. These data were taken to represent rates of filling of the gut, which apparently was able to lose 8.5 per cent of the body weight of the worm through successive handlings (Adolph, 1927) after it had remained undisturbed in water overnight.

However, it was found in the present experiments that, if both mouth and anus were

either ligated or stoppered with small glass plugs tied in place firmly with ligature thread (but not so tightly as to induce posterior autotomy) and a worm was allowed to come to gross weight overnight, it still lost an average of 13.4 ± 0.9 per cent of the basal body weight by handling. It is interesting to note that one individual was obtained which showed persistently high losses on handling, in one instance expelling 47 per cent of its basal weight at the time of handling.

It seemed that this loss of fluid was from the nephridiopores. This could be ascertained in anterior segments which possess no dorsal pores. On occasion too severe handling may have induced a *saignée* or draining reflex (Cuénot, 1898), whereby the worm suffered a loss of coelomic fluid from the dorsal pores locally, but this was not definitely established.

The contribution of the gut to water exchanges remained to be seen. In ordinary handling, little or no voiding of feces was observed. Voiding more often follows rough treatment. Lack of defecation was regarded as presumptive evidence of lack of appreciable voiding of water from the anus; and only minute quantities of fluid were observed to be cast from the mouth, this act also in part being dependent on the strenuousness of handling.

Jackson's (1925) histological studies on a single worm indicated that the intestinal lumen occupied about 15.2 per cent of the body volume. It is doubtful that ordinary handling could express such a volume of fluid, especially since the digestive tract is often laden with earth and mucus, undoubtedly requiring persistent peristalsis for their removal. No evidence for removal of such large amounts of feces was found.

With evidence for the nephridial path as a considerable factor in the water exchanges of the earthworm but with the existence also of a definite gut path, a quantitative estimation of the magnitude of each was attempted. To measure the excretion rate of the intestine, specially designed cannulas constructed of capillary tubing, calibrated for volume per unit length, were inserted into the mouth alone, while the anus was plugged (or vice versa); or one worm was allowed to excrete into a cannula at each end. The volume of fluid put out was thus determined at the level of the worm at intervals (Fig. 4). At 15° and 16° C. rates were found to be 0.22 ± 0.13 per cent of the mean gross weight per hour of 15 worms and bore no relation to body weight within the range of weights of worms used. A few manometric tests revealed that fluid was extruded into a mouth cannula until a maximal pressure of approximately 4.5 cm. of water was reached.

### NEPHRIDIAL OUTPUT

Figure 5 shows the apparent rate of gain of weight, falling with the frequency of handlings. This is believed to be due to the loss of fluid discharged upon dropping the worm into the tared cup. Adolph (1927) showed that similar manipulation could induce the expulsion of fluid. While the weight of the worm and its nephridial contents may thus be obtained at any given weighing, the procedure robs the worm of a fraction of its nephridial content so that the next period starts from a smaller load than is indicated by the point on the graph. However, the value for $\alpha$ for this procedure may not be the same as that for handling. The slope of the line between the point representing basal weight (abscissa) and the point representing the next weighing might be used as a minimal value for the rate of gain of weight of an undisturbed individual; and this may be regarded also as the rate of excretion of fluid by the nephridia into their reservoirs.

The rate of excretion thus determined is not found to vary with the interval of time between the first two points, suggesting that until the reservoir is filled, the rate of

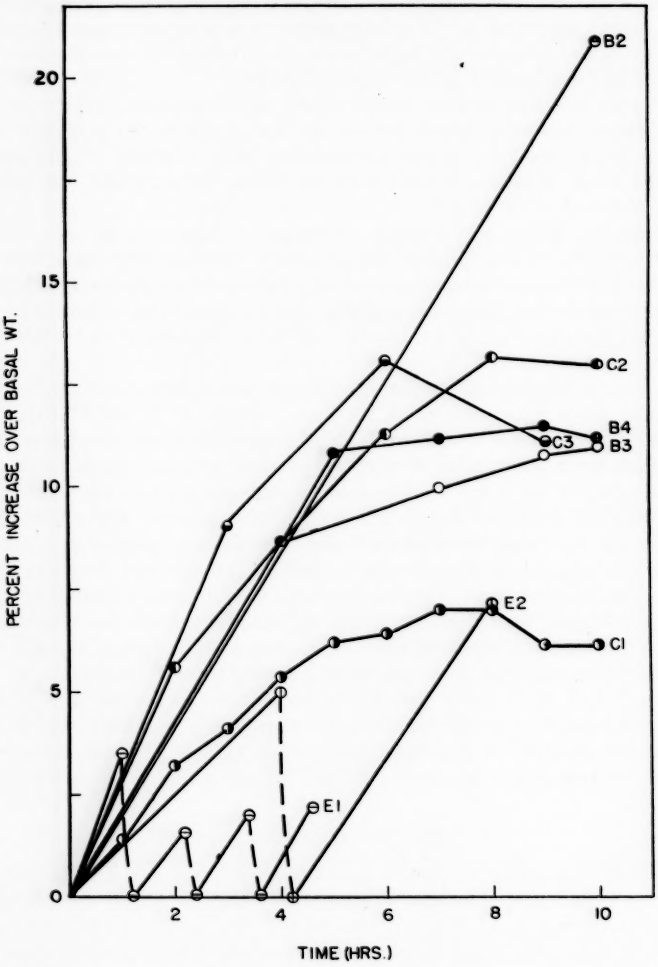


FIG. 5.—Intake rate of integument or excretion rate of nephridial battery may be determined from initial slopes. Broken lines indicate that the same individual was reduced to basal weight again. Worms are in water balance.

accumulation of fluid is constant. What happens when the reservoirs of undisturbed worms become filled has not been ascertained. Cuénot (1898) asserted that the reservoir is emptied about every 3 days in order that passage of coelomic fluid through the

excretory organ may be maintained, but there seems to be no firm basis for this view. The excretion rate might fall off, or there might be a periodic expulsion of fluid from the muscular duct. Such expulsions would conceivably play a part in giving rise to diurnal fluctuations.

In the above tests worms were weighed with adhering water and lifted carefully as described in the section on methods; it is likely that little initial expulsion of fluid is induced by this method of weighing.

The minimal rate of intake (and excretion) estimated from the initial slopes in 68 cases (on occasion the same worm was reduced to basal again after the first weighing and retested) averaged 2.55 ± 0.14 per cent of the basal weight per hour at 19° and 20° C. No significant differences were noted between worms having buccal and anal orifices either open or stoppered by plugs or ligation. In 41 cases where worms plugged or ligated at both ends were employed, the average rate was 2.49 ± 0.20 per cent of the basal weight per hour; and in 27 cases of unplugged specimens the rate was 2.84 ± 0.27 per cent. If a significant difference between the means of the two types of specimens had been manifested, it might be expected, as was actually found, that the plugged or ligated specimens would show lower rates, since tying off the extremities injures the region, probably eliminating the function of some nephridia, thus reducing the size of the effective battery of units.

## THE INTEGUMENT

In worms in water balance the total rate of output is equal to the rate of excretion of the nephridia plus that of the intestine, the larger portion being the nephridial one. If worms are dehydrated to varying degrees by exposure to air, they may, on replacement in water, regain body weight at rates varying with the water deficit (Fig. 6). This shows the ability of the integument to take in water more rapidly than nephridia eliminate it.

The plotting of Figure 6 requires further explanation. If we wish to distinguish rates at which the integument takes up water after worms suffer varying water losses, it is most convenient to compare them early in the recovery period, since they ultimately fall off to the normal turnover rate as the worms return to water balance. It has been shown that the recovery of water by dehydrated, isolated muscles is proportional to the square root of time for initial periods (Adolph, 1931; Wolf, 1940; and others). It appears from a few data that this also holds for dehydrated earthworms (Fig. 7). For Figure 6 an arbitrary recovery time (49 min.) for dehydrated specimens was chosen, as well as an arbitrary degree of dehydration, so that complete recovery would not be attained in the given time, and most dehydrations permitted viability (Jackson, 1925).

So long as

$$I = k\sqrt{t}, \tag{1}$$

where $I$ is the intake in percentage of basal weight in $t$ minutes and $k$ the constant of proportionality,

$$k = \frac{I}{\sqrt{t}} = \frac{I}{7}. \tag{2}$$

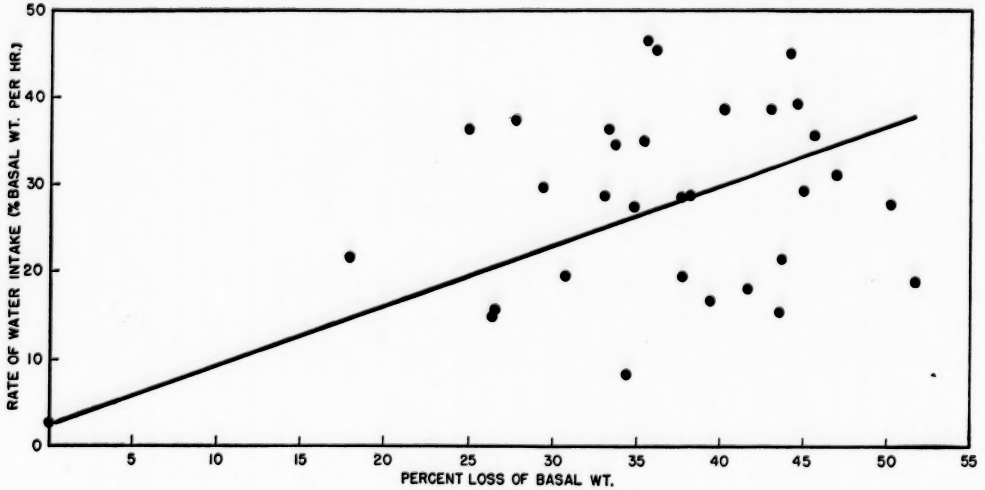


FIG. 6.—Net rate of intake of water through the integument at ninth minute of recovery. The point at zero per cent loss of weight represents the mean excretion rate of the nephridia determined in water balance from 68 cases.

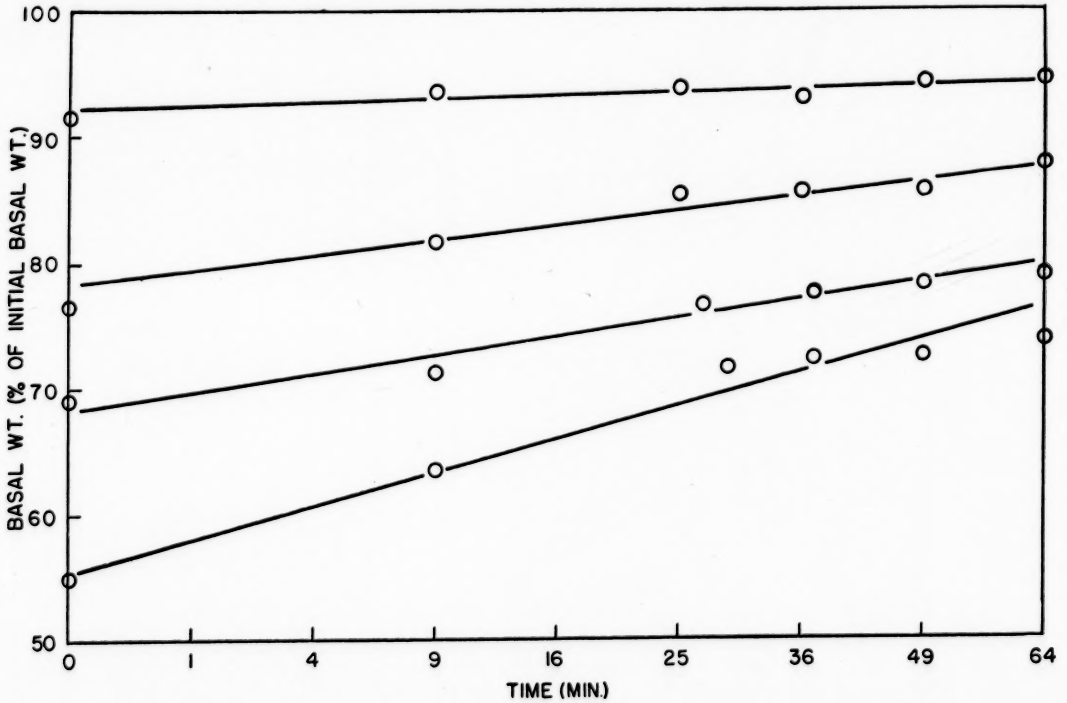


FIG. 7.—The weight of worms recovering from different levels of dehydration is proportional to $\sqrt{t}$. The points of each curve represent the mean weight of 3 worms.

The intake per minute at the ninth minute of the recovery period is, therefore,

$$\frac{dI}{dt} = \frac{k}{2\sqrt{t}} = \frac{I}{7 \times 2\sqrt{t}} = \frac{I}{7 \times 2\sqrt{9}} \tag{3}$$

or, per hour,

$$\frac{dI}{dt} = \frac{I \times 60}{7 \times 2 \times 3} = 1.43\, I. \tag{4}$$

The slopes at 9 minutes were chosen simply as conveniently large ones for comparison. The initial slope (at $t = 0$), being infinity, attests to the inadequacy of equation

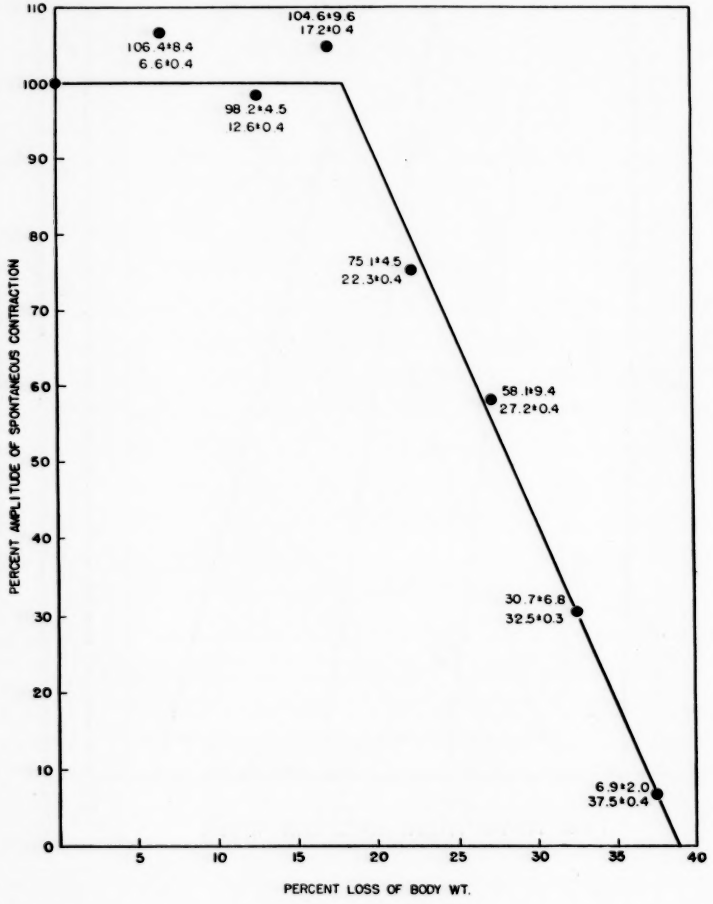


FIG. 8.—Relation between the amplitude of spontaneous contractions and the loss of body weight which obtained under the conditions stated in the text. Values of the ordinate and abscissa are given with their standard errors for 9 worms.

(1), and that slope obviously cannot be used. At zero dehydration under the conditions of these experiments the net intake of the integument is equal to the nephridial output (the worm is in water balance), i.e.,

$$I = ct\,, \tag{5}$$

where

$$c = 2.55 \text{ per cent per hour}\,.$$

Therefore,

$$\frac{dI}{dt} = c = 2.55 \text{ per cent of the basal body weight per hour at all times}\,. \tag{6}$$

Although a worm is reduced to basal weight, it is not dehydrated with respect to tissue water; so it is believed that basal weight represents the same water balance as gross weight. The integument seemed to vary its rate of intake only in response to dehydration of body tissue.

## SPONTANEOUS CONTRACTIONS

It was thought interesting to study some activity in the earthworm which might be related to the state of tissue dehydration and hence to the rate at which the integument takes in water. The activity chosen was measured by recording on a kymograph the amplitude of the spontaneous longitudinal contractions of a worm's body when suspended by threads passed through one of the anterior and one of the posterior segments. The anterior end was suspended uppermost. Handling of the worm during threading tends to reduce the worm to basal weight, so that evaporation is largely loss of tissue water. The tension applied by the recording lever of the kymograph was approximately 0.3 gm. The amplitude of the contractions was measured by the distance between the highest and the lowest of the points obtained on the kymogram in a 2-minute run which was taken every 20 minutes, at which times the worm was weighed. Figure 8 indicates the relation between the amplitude of these contractions and the loss of body weight. There is no evidence that the same curve holds for worms recovering from water deficits.

## DISCUSSION

The excretion rate of the intestine is obtained in terms of basal weight by multiplying the rates, which above were based on gross weight, by the factor 113.4/100.0 (13.4 per cent is the amount of fluid lost in reducing worms to basal weight). The value is 0.25 per cent of the basal body weight per hour. This amounts to only one-tenth of the rate for the sum of the nephridial units plus the rate of any gut excretion which may go on under these conditions, and it becomes apparent that the water exchanges of the two systems are of different orders of magnitude. The temperatures at which gut and nephridial determinations were made were not strictly alike. It is possible that the gut is not concerned primarily with maintenance of water balance but rather with the formation of digestive juices or the rendering of the feces fluid, in order that, as Darwin (1881) has indicated, the excrement may be spread about the burrow walls by the posterior end, which is used much as a trowel.

When a worm is reduced to basal weight, it merely suffers loss of a dead water load. Its removal reduces body volume. Since it is believed that the volume lost has come from

the lumina of the nephridia, we can, from the number of nephridia per specimen, estimate the mean capacity of the muscular duct plus any other part of the nephridium which may act as a storage place for dischargeable fluid (the ampulla may serve in some measure). The number of segments in 7 adult worms, ranging from 2.42 to 6.93 gm. basal weight, averaged 143 ± 4. The number of nephridia per worm may be expressed as $N = 2s - 8$, where $s$ represents the number of segments. $N$ therefore averages 278 nephridia. Knowing the amount of fluid expelled when the average worm is reduced from gross to basal weight (13.4 per cent), we can determine the capacity of the storage space of an average nephridium as 13.4 per cent ÷ 278 or 0.048 per cent of the basal body weight, which in a 4-gm. worm constitutes a volume of 1.9 cmm. Gegenbaur (1853) gives plates and dimensions of *Lumbricus* nephridia in worms of unstated sizes which could accommodate this volume of fluid. Since the reservoir is effectively cylindrical, the volume varies as the square of the radius, so that with small changes of radius, comparatively large changes of content may be accommodated. A few observations by the writer corroborate the hypothesis that the nephridial reservoir is capable of holding the predicted water stores.

The average length of time required by a basal worm to attain gross weight is found from the figures for average loss from gross to basal weight and the nephridia excretion rate,

$$13.4 \text{ per cent} \div \frac{2.55 \text{ per cent}}{\text{hour}} = 5.25 \text{ hours} .$$

Possible factors affecting diurnal fluctuations of gross and basal weights may be summarized as (1) reservoirs varying in content as a result of periodic, partial expulsion of liquid; (2) loss of salts to distilled water, accompanied by loss of water, leading to a downward weight trend; (3) intestinal excretion, the effect of which may have been seen in the fluctuations of basal worms. The average excretion rate of the intestine (0.25 per cent per hour) could by accumulating in the gut give rise to approximately a 6 per cent weight increase in 24 hours, while the average change of basal weight seen in this time was only 2.3 per cent, an amount easily accounted for by intestinal fill; (4) small losses from the gut or body cavity in handling; (5) failure to attain the same degree of discharge from the nephridial battery in successive reductions to basal weight.

It was early thought that the nephridia served respiratory functions, Gegenbaur (1853) first suggesting their excretory role. But the nephridia may play an indirect part in respiration by keeping the integument moist. It may generally be observed that clusters of worms found in earth are quite moist. It has been held that exudation of coelomic fluid (and mucus glands) provides moisture, but it would seem more likely that coelomic-fluid exudation rather depends on irritating influences. If, during wet weather, earthworms store fluid in the nephridia, fractions of this might then be released at subsequent times. If the volume of fluid which may be contained in the gut is of the order of half of the gut volume (the remainder of the gut containing earth and food), the possible total store is 13 per cent + 8 per cent, or one-fifth of the body weight, or about one-quarter of the actual tissue water in the body. No data were collected to show the effect on the excretion rate or the storage space of the salts and other osmotic pressure-exerting substances which may exist in soils.

It is important to know the error which may be introduced by the use of gross, rather

than of basal, weights; but at the same time it should be stressed that the weight of a basal worm, too, is actually made up partly of an unknown dead water load in the gut which may be 15 per cent of the body weight.

The spontaneous contractions during dehydration represent part of a syndrome of dehydration described by Wolf (1938). The "rolling" phase in which elements of contracted longitudinal musculature travel in a circular sense about the body was here observed, and when a worm was lifted from its support and placed on a substratum, active rolling ensued.

## SUMMARY

1. The mean diurnal fluctuations of gross and basal weights are 3.2 ± 0.3 and 2.3 ± 0.3 per cent of basal body weight, respectively. Both weights show a parallel downward trend in distilled water, over periods of 16 days, which may be due to permanent loss of salts and water during starvation.

2. The fraction ($L_n$) of fluid remaining in the nephridial reservoirs after $n$ handlings was found to be given by $L_n = L_i\,(1-\alpha)^n$, where $L_i$ is the initial content and $\alpha$ had the value 0.631.

3. At 15° and 16° C. the gut was found to excrete fluid at approximately 0.25 per cent of a worm's basal body weight per hour, and this could be extruded through a mouth cannula until a maximum pressure of approximately 4.5 cm. of water was attained.

4. The gut was found not to contribute to any appreciable loss of weight, in worms undisturbed in water for some time, upon subsequent handling.

5. At 19° and 20° C. the nephridial battery of an earthworm was found to excrete fluid at a minimal rate of 2.55 ± 0.14 per cent of the basal body weight per hour, approximately ten times the rate of the gut.

6. The average difference between gross and basal weight amounted to 13.4 ± 0.9 per cent of the basal body weight. On this basis the storage capacity of an average nephridium may be estimated as 0.048 per cent of the basal body weight.

7. In worms in water the rate of net intake by the integument at given times increases with deficits of body water previously imposed by desiccation in air.

8. The amplitude of spontaneous contractions is unaffected by robbing a worm of as much as 18 per cent of its body weight of water, but it falls off in direct proportion thereafter, as the water loss progresses until no movement is evident when about 40 per cent of body weight has been lost.

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# METHODS FOR EVALUATION OF HEAD TYPES IN PLANARIANS

(Twelve figures)

WILLIAM A. CASTLE
Brown University and University of Chicago

Students of regeneration have for many years been confronted with the problem of assigning a quantitative measure to the anterior structures resulting from the regulation of experimentally isolated fragments of the body of triclad flatworms. In some of the American species the reaction appears to be of an "all-or-none" type (Buchanan, 1933, 1936, 1938) in that either normal heads are regenerated or none at all, while in planarians the regenerating fragments form a series of anterior structures graded from the normal head to a complete absence of any headlike organ or new tissue. Child (1911, 1912) distinguished five types of these anterior regenerated structures: normal; teratophthalmic, in which the head is of normal shape but the eyes are either decidedly asymmetrical in size or exhibit various degrees of fusion; teratomorphic, in which there is a single median eye and in which the shape of the head varies from the normal with laterally or anterolaterally placed auricles to a pointed anterior outgrowth; anophthalmic, without eyes but possessing a pointed outgrowth of new tissue; and acephalic or headless, in which there is no outgrowth of new tissue, the anterior end being rounded and the wound contracted and filled with scar tissue. These types of regenerates have been illustrated many times in the publications of Child and of his students.

It has been adequately demonstrated by a number of workers (Buchanan, 1922; Hinrichs, 1924; Rulon, 1936, 1937, 1938; Miller, 1937) that these types of anterior outgrowths form a more or less continuous series, for agents or conditions which favor the formation of heads shift the observed experimental outgrowths toward the upper (normal) end of the scale, while agents or conditions which inhibit head formation shift the observed types toward the lower (acephalic) end of the scale.

The concept of "head frequency" as an expression of the result of regeneration was given a roughly quantitative measure when Child and his students assigned to the head types arbitrary values. Various scales have been used: 100, 80, 60, 40, 20; 50, 40, 30, 20, 10; 5, 4, 3, 2, 1; etc., differing only in the choice of scale units, all of them identical in that in each one the successive steps in inhibition of a head are separated by equal intervals along an arithmetic scale. The use of these scales has made possible the derivation of a single, numerical expression for the result of an experiment involving numbers of pieces by simply taking as the "head-frequency index" the numerical average of the arbitrary values assigned to the types of heads regenerated. Thus, in using a scale of 50, 40, 30, 20, 10, a head-frequency index of 50 would indicate that all pieces had regenerated normal heads, one of 10 that all pieces were acephalic, and one of 35 that the "average" head resulting was intermediate between the teratomorphic and teratophthalmic types.

Valuable as this scheme has been in making possible a roughly quantitative estimate of the result of anterior regeneration of planarian fragments, it remains in some degree unsatisfactory as long as the numerical values assigned are arbitrary. The assigning of

numerical values with equal intervals between the values for successive categories implies that each head type differs from the one above or below it in the scale in some mathematical fashion. If this is true, the relationship may be interpreted theoretically by some such graphical situation as that suggested in Figure 1. The relationship here illustrated is such that equal advances in conditions favoring head formation elicit responses in anterior outgrowths represented by equal successive steps in the perfection of heads.

If the above hypothesis is true, then under conditions that permit the formation of all types of heads the intermediate categories will be well represented. That such is not the case is suggested by the observations of different workers. Results published by

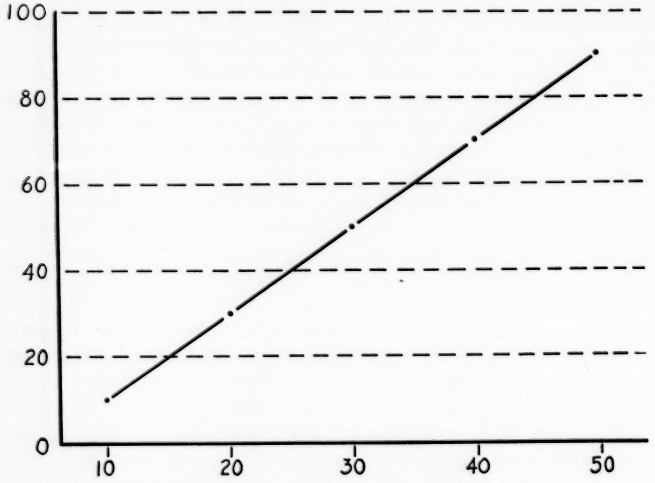



FIG. 1.—Diagram of the hypothetical relationship between head-frequency indices (abscissas) of the equal-interval scale and the ranges of the head types (ordinates), based on the assumption that the ranges for the different head types are equal. The ranges are shown on a scale of 0 to 100; 0–20, acephalic; 20–40, anophthalmic; 40–60, teratomorphic; 60–80, teratophthalmic; 80–100, normal.

Sivickis (1923) and Buchanan (1938) are characteristic of the experience of others. In the tabulations of the types of heads observed by these two workers it is clear that anophthalmic outgrowths occur in fewer numbers than do normals, teratophthalmics, or acephalics and that teratomorphic heads are observed relatively infrequently.

It is obvious that to be of most use in expressing head frequency accurately the numerical scale adopted must bear some relation to the size of the groups represented. The use of an equal-interval scale would be justified if the same change in experimental condition that shifts normal heads to teratophthalmic also shifted teratophthalmic to teratomorphic and so on down the scale. If this relationship as revealed by the observed frequency of occurrence of heads of different types does not exist, then a revision of the indices should be made, and the types of heads occurring in fewer than the expected numbers should be allotted narrower "bands" in the entire scale. The numerical steps between successive grades in head perfection (or inhibition) will then be unequal.

The writer, in being confronted with the evaluation of the results of regeneration of large numbers of fragments of *Dugesia dorotocephala* (=*Euplanaria dorotocephala*) and *D. tigrina* (=*E. maculata*),[1] has been impressed not only by the inequality in numbers of individuals occurring in the different recognizable categories but also by the inadequacy of the scale of arbitrarily assigned values for discriminating between experiments in which the differences in head frequency are slight or not obvious. It is this experience that has led to the present attempt to develop more satisfactory methods for the evaluation of head-frequency data.

## EXPERIMENTAL OBSERVATIONS

As a consequence of a series of regeneration experiments undertaken on *D. dorotocephala* and *D. tigrina*, records of anterior structures formed by considerable numbers of fragments of members of these two species have been made available. Fragments used

TABLE 1

RECORD OF AN EXPERIMENT WITH *D. dorotocephala* TO SHOW DISTRIBUTION OF REGENERATED STRUCTURES TYPICALLY OBSERVED

(Size of pieces: one-eighth of 12–13-mm. worms after removal of heads and tails)

| Level | Number of Pieces | Normal | Teratoph-thalmic | Tera-to-morphic | Anoph-thalmic | Acephalic | Dead |
|---|---|---|---|---|---|---|---|
| A | 10 | 8 | 2 | ..... | ..... | ..... | ..... |
| B | 10 | 2 | 4 | ..... | 2 | 2 | ..... |
| C | 10 | ..... | 1 | 1 | 4 | 4 | ..... |
| D | 10 | ..... | ..... | 1 | 2 | 7 | ..... |
| E | 10 | ..... | 6 | 1 | ..... | 3 | ..... |
| F | 10 | 1 | 1 | 1 | 3 | 4 | ..... |
| G | 10 | 1 | 4 | 1 | 2 | 2 | ..... |
| H | 10 | 6 | 4 | ..... | ..... | ..... | ..... |

were one-eighth of the length of the body after removal of the head and tail; specimens of *D. dorotocephala* used were approximately 12–13 mm. in length, and of *D. tigrina* approximately 10–11 mm. Fragments were considered in units of 10 from each of the eight levels of the body; the 80 fragments which resulted from cutting up 10 worms constituted an experiment. Records were kept on this basis. Data are here considered from 60 experiments with *D. dorotocephala* (a total of about 4,800 fragments) and about 150 experiments with *D. tigrina* (a total of about 12,000 fragments).

A representative record of a single experiment with *D. dorotocephala* is shown in Table 1. It is to be observed that at levels of the body showing high head frequency (levels A and H) the resulting anterior outgrowths as determined after 10 days are all of the higher orders of heads, usually distributed between normals and teratophthalmics. At levels showing lowest head frequency (level D) acephalic forms predominate. Occasionally, however, there are to be found units of 10 pieces from the same level of the body and isolated under as nearly as possible the same experimental conditions in which both extremes of the head-type scale are represented, together with a scattering of heads of intermediate type (levels B, F, and G). The conditions under which these

[1] See Hyman (1939) for the taxonomic history of these two species.

fragments have regenerated have been such that they have permitted the formation of all types of outgrowths which Child has grouped into his five categories.

From the records of the experiments all units of 10 pieces which have shown the two extremes of the range from acephalic to normal have been selected for use in the calculations to be discussed under Method I, without regard to the fact that in many of these units one or more of the intermediate head types may not be represented (Table 1, level B, for example). These have been totaled separately for *D. dorotocephala* and *D. tigrina*, with the result tabulated in Table 2. For *D. dorotocephala* a total of

TABLE 2

DISTRIBUTION OF HEAD TYPES IN EXPERIMENTAL UNITS SHOWING RANGE FROM NORMAL TO ACEPHALIC

| HEAD TYPE | *D. dorotocephala* | | *D. tigrina* | |
|---|---|---|---|---|
| | Number | Per Cent | Number | Per Cent |
| Acephalic | 424 | 35.01 | 551 | 20.18 |
| Anophthalmic | 99 | 8.18 | 446 | 16.34 |
| Teratomorphic | 72 | 5.95 | 153 | 5.60 |
| Teratophthalmic | 356 | 29.40 | 613 | 22.45 |
| Normal | 260 | 21.47 | 967 | 35.42 |
| Total | 1,211 | 100.01 | 2,730 | 99.99 |

1,211 regenerated fragments are included in this tabulation. Of these 424, or 35.01 per cent, are acephalic; 99, or 8.18 per cent, are anophthalmic; 72, or 5.95 per cent, are teratomorphic; 356, or 29.40 per cent, are teratophthalmic; and 260, or 21.47 per cent, are normal heads. In the tabulation for *D. tigrina* 2,730 fragments are included, with the distribution of regenerated structures as shown in Table 2. It is obvious from these data as from the records of Sivickis (1923) and Buchanan (1938) that the anophthalmic and teratomorphic heads occur with much lower frequency than do the other types.

## NEW METHODS FOR EXPRESSION OF HEAD FREQUENCY

### METHOD I

The method here presented is a means for assigning definite values to heads of different types. These indices differ from those of the older scale in that they are assigned on the basis of the observed frequency of occurrence of the head types in experimental units showing the whole range of the scale. The indices are not separated by equal intervals along the scale but by distances determined by the widths of the ranges of the different categories.

In pieces isolated from the same level of different worms from a homogeneous stock individual and uncontrollable differences in physiologic condition of the worms, in the size of the piece, or in the conditions in the immediate vicinity of the regenerating fragments or other factors appear to be sufficient to account for the variation in the types of heads regenerated. If each factor acting has the same effect at all levels of the scale, then the frequency distribution of regenerated structures should be that of a normal probability curve, the acephalic and normal forms occupying opposite extremes of the

curve and the intermediate classes of heads lying between. It is further assumed that the thresholds between successive grades of head development are at relatively fixed points on the scale. Such a distribution of the data for *D. dorotocephala* found in Table 2 is presented in Figure 2.

From the available information on the percentages of the total population found in the respective classes it is possible to calculate, in terms of the standard deviation or in terms of an arbitrary scale of 0–100 units assigned to the total range, the limits (thresholds) and class ranges of the five separate classes of heads. For these calculations the

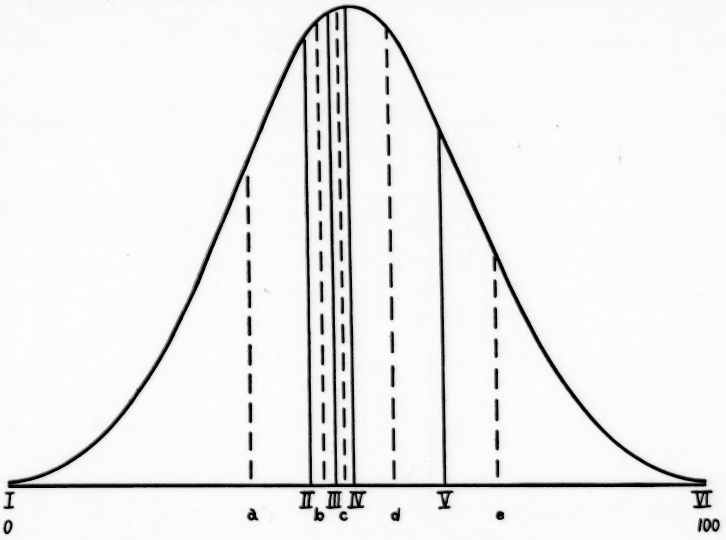



FIG. 2.—Observed frequency distribution of head types in *D. dorotocephala* fitted to a normal probability curve. The distribution is based on a compilation of results from units of 10 pieces which show the whole range of head types, selected from the author's experiments. Solid lines represent the limits or thresholds between successive classes of head types; broken lines represent the class medians. Values for the abscissas of the class limits (I, II, . . . . , VI) are given in Table 3 in terms of standard deviations and in terms of the scale of 0–100 assigned to the entire range. Values for the abscissas of the class medians (*a*, *b*, . . . . , *e*) are given in Table 4.

normal-curve area table in Chaddock (1925, p. 463) has been used. The values given in this table are such that 3.1 standard deviations from the mean include 49.93 per cent of the cases. This distance, measured both plus and minus from the mean, will therefore include 99.86 per cent or practically all the cases. This total range of 6.2 standard deviations is taken as equal to the 100 units of the arbitrary scale in which the final values are to be expressed.

The calculations for the procedure outlined below are set forth in Table 3. In column 2 are recorded the percentages of the total population present in each class. From these values the running sums ($\Sigma p$) shown in column 3 are derived. Then 50 is subtracted from each of these values ($\Sigma p - 50$) to obtain the percentages between the mean and

the given threshold. These values are shown in column 4, the sign indicating on which side of the median each threshold lies. The abscissa of the unit normal probability curve, or inverse probability function ($prf^{-1}$), for each of these thresholds is determined in terms of the standard deviation (col. 5) by reference to a normal probability-curve area table such as that cited in Chaddock. The algebraic differences between the successive values in column 5 are recorded in column 6. These differences represent in terms of standard deviations the ranges of the classes. In column 7 these ranges are converted into the units of the arbitrary scale of 0–100 by multiplying them by 100/6.2. Finally, by finding in column 8 the running sums of the values in column 7, the class limits or

TABLE 3

CALCULATION OF CLASS LIMITS AND RANGES

(*D. dorotocephala*)

| (1) Class | (2) Percentage in Each Class $p$ | (3) Running Sum of (2) $\Sigma p$ | (4) Distance from Mean $\Sigma p - 50$ | (5) Inverse Probability Function $prf^{-1}(4)$ | (6) Class Range in $\sigma$ | (7) Class Range in Scale Units (6) $\frac{100}{6.2}$ | (8) Class Limits from Running Sum of (7) |
|---|---|---|---|---|---|---|---|
| | | (0.00) | (−50.00) | $-\infty(-3.1)$ | | | 0.00 (I) |
| Acephalic | 35.01 | | | | 2.715 | 43.79 | |
| | | 35.01 | −14.99 | −0.385 | | | 43.79 (II) |
| Anophthalmic | 8.18 | | | | 0.214 | 3.45 | |
| | | 43.19 | − 6.81 | −0.171 | | | 47.24 (III) |
| Teratomorphic | 5.95 | | | | 0.150 | 2.42 | |
| | | 49.14 | − 0.86 | −0.021 | | | 49.66 (IV) |
| Teratophthalmic | 29.40 | | | | 0.812 | 13.10 | |
| | | 78.54 | +28.54 | +0.791 | | | 62.76 (V) |
| Normal | 21.47 | | | | 2.309 | 37.24 | |
| | | 100.01 | +50.01 | $+\infty(+3.1)$ | | | 100.00 (VI) |
| Total | 100.01 | ..... | ..... | ..... | 6.200 | 100.00 | ..... |

thresholds are determined. The Roman numerals (I, II, . . . . , VI) accompanying these values refer to the positions of these limits as indicated in Figure 2.

While the determinations outlined above locate the limits of the five classes with respect to the total range of the population, the values are in some ways not suitable for use in the expression of head frequency. For this purpose a value is desired which for any one class is more representative of the entire class than is the value for either of the thresholds limiting the class. Although not as constant a value as those for the thresholds determined above, the value for the median of each class has been chosen for this purpose. The reasons for this choice will be indicated more fully in a later section of this paper. The abscissas for the medians in terms of the arbitrary scale are determined from the class-percentage frequencies by calculations indicated in Table 4. From the

class percentages the medians are located in the population by finding the percentages of cases lying below the successive medians (col. 3). Then 50 is subtracted from each

TABLE 4

CALCULATION OF ABSCISSAS OF CLASS MEDIANS

(*D. dorotocephala*)

| (1) Class | (2) Percentage in Each Class $p$ | (3) Percentage below Successive Medians | (4) Distance from Median (3)−50 | (5) Inverse Probability Function $prf^{-1}(4)$ | (6) Abscissa in $\sigma$ (5)+3.1 | (7) Abscissa in Scale Units (6) $\frac{100}{6.2}$ |
|---|---|---|---|---|---|---|
| Acephalic | 35.01 | 17.55 | −32.45 | −0.933 | 2.167 | 34.95(*a*) |
| Anophthalmic | 8.18 | 39.10 | −10.90 | −0.277 | 2.823 | 45.53(*b*) |
| Teratomorphic | 5.95 | 46.17 | − 3.83 | −0.096 | 3.004 | 48.45(*c*) |
| Teratophthalmic | 29.40 | 63.84 | +13.84 | +0.354 | 3.454 | 55.71(*d*) |
| Normal | 21.47 | 89.24 | +39.24 | +1.240 | 4.340 | 70.00(*e*) |

of these values to locate them with respect to the median for the entire population (col. 4). By reference to the normal-curve area table (Chaddock, 1925) the inverse prob-

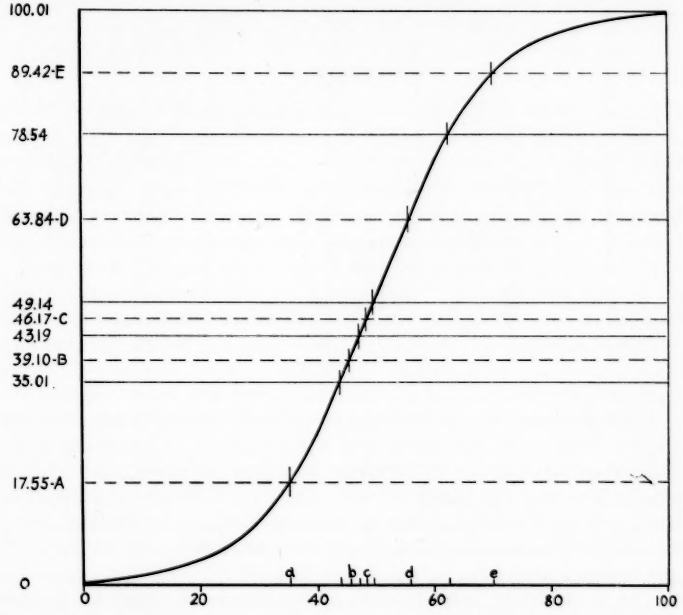



FIG. 3.—Relationship between observed percentage distribution of head types (ordinates) and values determined by Method I for the class limits and medians (abscissas). Values for the abscissas of the class limits (I, II, . . . . , VI) are given in Table 3 and for the medians (*a*, *b*, . . . . , *e*) in Table 4.

ability function of each of these points is found (col. 5), thus expressing the locations in terms of standard deviations from the mean. The addition of 3.1 to these values (col. 6) locates the individual class medians with respect to the extreme lower end of the scale, and these values are converted to the arbitrary-scale units by multiplying them by 100/6.2 (col. 7). The key letters ($a$, $b$, . . . . , $e$) accompanying the values in this column refer to the locations indicated in Figure 2.

Using the values determined above for the abscissas of the class limits and medians, a curve may be constructed to show their relationship to the percentage distribution of head types. This curve is shown in Figure 3 and is to be contrasted to the hypothetical

TABLE 5

CALCULATION OF CLASS LIMITS AND RANGES

(*D. tigrina*)

| (1) Class | (2) Percentage in Each Class $p$ | (3) Running Sum of (2) $\Sigma p$ | (4) Distance from Mean $\Sigma p - 50$ | (5) Inverse Probability Function $prf^{-1}(4)$ | (6) Class Range in $\sigma$ $\Delta$ | (7) Class Range in Scale Units (6) $\frac{100}{6.2}$ | (8) Class Limits from Running Sum of (7) |
|---|---|---|---|---|---|---|---|
| | | (0.00) | (−50.00) | $-\infty(-3.1)$ | | | 0.00 |
| Acephalic | 20.18 | | | | 2.265 | 36.53 | |
| | | 20.18 | −29.82 | −0.835 | | | 36.53 |
| Anophthalmic | 16.34 | | | | 0.490 | 7.90 | |
| | | 36.52 | −13.48 | −0.345 | | | 44.43 |
| Teratomorphic | 5.60 | | | | 0.146 | 2.35 | |
| | | 42.12 | − 7.88 | −0.199 | | | 46.78 |
| Teratophthalmic | 22.45 | | | | 0.573 | 9.24 | |
| | | 64.57 | +14.57 | +0.374 | | | 56.02 |
| Normal | 35.42 | | | | 2.726 | 43.97 | |
| | | 99.99 | +49.99 | $+\infty(+3.1)$ | | | 99.99 |
| Total | 99.99 | | | | 6.200 | 99.99 | |

situation suggested in Figure 1. Since in Figure 1 the intervals between successive values assigned to the head types are equal and the distances between the thresholds are represented as of equal size, the curve expressing the relationship between the two is a straight line. In Figure 3 the bands for inclusion of the classes correspond in width to the experimentally observed frequency of occurrence of the head types, the values for the medians and for the thresholds are as determined above, and the curve of relationship is of the sigmoid type.

From the data recorded in Table 2 for *D. tigrina* a series of values for the class ranges, limits, and the medians has been determined for this species. The method of procedure is precisely the same as has been outlined above for *D. dorotocephala*. The calculations and final values are shown in Tables 5 and 6. Since the percentage distribution of the

head types differs from that for *D. dorotocephala*, the values are not the same as for *D. dorotocephala*—a result to be anticipated. If the values determined for *D. tigrina* are plotted against the class frequencies, the points will fall upon the same curve as

TABLE 6

CALCULATION OF ABSCISSAS OF CLASS MEDIANS

(*D. tigrina*)

| (1) Class | (2) Percentage in Each Class $p$ | (3) Percentage below Successive Medians | (4) Distance from Median (3)−50 | (5) Inverse Probability Function $prf^{-1}(4)$ | (6) Abscissa in $\sigma$ (5)+3.1 | (7) Abscissa in Scale Units (6) $\frac{100}{6.2}$ |
|---|---|---|---|---|---|---|
| Acephalic | 20.18 | 10.09 | −39.91 | −1.276 | 1.824 | 29.42 |
| Anophthalmic | 16.34 | 28.35 | −21.65 | −0.572 | 2.528 | 40.78 |
| Teratomorphic | 5.60 | 39.32 | −10.68 | −0.271 | 2.829 | 45.63 |
| Teratophthalmic | 22.45 | 53.55 | + 3.55 | +0.089 | 3.189 | 51.43 |
| Normal | 35.42 | 82.28 | +32.28 | +0.926 | 4.026 | 64.92 |

that shown for *D. dorotocephala* in Figure 3. This must of necessity be so, since both series of values are determined in the same fashion from constants for a normal probability curve and since the curve of relationship has a definite mathematical relationship to the normal curve.

METHOD II

The indices developed in the preceding section depend for their determination upon the availability of a body of data from experiments in which the individual groups of pieces are small. Wright (private communication) has applied to the problem an elaboration of the method he has used for the determination of values for the thresholds between different degrees of polydactyly in guinea pigs (Wright, 1934). His method makes possible the determination of values for the ranges and limits of the three intermediate classes of heads from data on experiments with large numbers of pieces from a homogeneous stock. Having made this determination, it is then possible to describe the population of regenerated structures by locating its mean relative to the total range of the three classes and by determining its variability by finding the standard deviation from this mean.

The assumptions underlying the method are the same as those underlying Method I, namely, (1) that the frequency distribution of head types should be that of a normal probability curve relative to the scale of factor combinations determining head types and (2) that the thresholds between successive grades of head development are at fixed points on the scale.

Wright applied the method to data on *D. dorotocephala* from the observations of several students. From the three different bodies of data the values determined for the class ranges were averaged after proper weighting to take into account the reliability of the different determinations. The final estimate was that the ranges of the three classes considered (anophthalmia, teratomorphia, and teratophthalmia) were in the proportion of 0.36:0.14:0.50, expressed as fractions of the total range of the three classes.

It is of interest to apply the method suggested by Wright to the whole of the mass of data from which a selection was made for use in the determinations of Method I. The writer wishes at this point to acknowledge his indebtedness to Dr. Sewall Wright for his painstaking care in explaining the method and for his permission to make use of the unpublished work.

In Table 7 are summarized the results from the 60 separate experiments on *D. dorotocephala* previously cited. Each experiment consisted of an original 80 pieces from 10 worms, 10 pieces from each of the 8 levels of the body indicated. The numerical and

TABLE 7

DISTRIBUTION OF HEAD TYPES: TOTALS FOR PIECES SURVIVING FROM SIXTY EXPERIMENTS

(*D. dorotocephala*)

| Level | | Normal | Teratoph-thalmic | Tera-to-morphic | Anoph-thalmic | Acephalic | Total |
|---|---|---|---|---|---|---|---|
| A | No. | 280 | 240 | 12 | 2 | 10 | 544 |
| | Per cent. | 51.47 | 44.12 | 2.21 | 0.37 | 1.84 | 100.01 |
| B | No. | 60 | 243 | 36 | 51 | 183 | 573 |
| | Per cent. | 10.47 | 42.41 | 6.28 | 8.90 | 31.94 | 100.00 |
| C | No. | 7 | 52 | 34 | 94 | 376 | 563 |
| | Per cent. | 1.24 | 9.24 | 6.04 | 16.69 | 66.78 | 99.99 |
| D | No. | 9 | 23 | 23 | 40 | 469 | 564 |
| | Per cent. | 1.60 | 4.08 | 4.08 | 7.09 | 83.16 | 100.01 |
| E | No. | 15 | 81 | 47 | 71 | 358 | 572 |
| | Per cent. | 2.62 | 14.16 | 8.22 | 12.41 | 62.59 | 100.00 |
| F | No. | 10 | 85 | 41 | 77 | 367 | 580 |
| | Per cent. | 1.72 | 14.66 | 7.07 | 13.28 | 63.28 | 100.01 |
| G | No. | 51 | 162 | 45 | 81 | 258 | 597 |
| | Per cent. | 8.54 | 27.14 | 7.54 | 13.57 | 43.22 | 100.01 |
| H | No. | 281 | 232 | 10 | 13 | 25 | 561 |
| | Per cent. | 50.09 | 41.35 | 1.78 | 2.32 | 4.46 | 100.00 |

percentage distribution of head types formed by the survivors from the original 600 pieces at each of the 8 levels are shown.

From the data presented in Table 7 estimates are made of the ranges of the five categories for each of the eight levels. For the sake of brevity in the tabulation and in the discussion to follow, these categories have been designated as *A*, acephalic; *B*, anophthalmic; *C*, teratomorphic; *D*, teratophthalmic; and *E*, normal. From the running sum of the percentages in each class 50 is subtracted to obtain the percentage between the mean and the given threshold. The abscissas of the unit normal probability curve (inverse probability function) corresponding to each of these frequencies is determined from a normal-curve area table (Chaddock, 1925). The difference between successive

values, taking account of signs, is the required class range in terms of the standard deviation. This procedure is the same as that used in Method I. The calculations are indicated in Table 8.

TABLE 8

ESTIMATION OF CLASS RANGES

(*D. dorotocephala*)

| Level of Body | Class | Percentage in Class $p$ | Summation of Percentages $\Sigma p$ | Distance from Median $\Sigma p - 50$ | Inverse Probability Function $prf^{-1}(\Sigma p - 50)$ | Range |
|---|---|---|---|---|---|---|
| A..... | *A* | 1.84 | 1.84 | −48.16 | −2.088 | 1.012 |
| | *B* | 0.37 | 2.21 | −47.79 | −2.012 | 0.076 |
| | *C* | 2.21 | 4.42 | −45.58 | −1.704 | 0.308 |
| | *D* | 44.12 | 48.54 | − 1.46 | −0.036 | 1.668 |
| | *E* | 51.47 | 100.01 | +50.01 | +3.1 | 3.136 |
| B..... | *A* | 31.94 | 31.94 | −18.06 | −0.469 | 2.631 |
| | *B* | 8.90 | 40.84 | − 9.16 | −0.232 | 0.237 |
| | *C* | 6.28 | 47.12 | − 2.88 | −0.073 | 0.159 |
| | *D* | 42.41 | 89.53 | +39.53 | +1.255 | 1.328 |
| | *E* | 10.47 | 100.00 | +50.00 | +3.1 | 1.845 |
| C..... | *A* | 66.78 | 66.78 | +16.78 | +0.434 | 3.534 |
| | *B* | 16.69 | 83.47 | +33.47 | +0.974 | 0.540 |
| | *C* | 6.04 | 89.51 | +39.51 | +1.254 | 0.280 |
| | *D* | 9.24 | 98.75 | +48.75 | +2.240 | 0.986 |
| | *E* | 1.24 | 99.99 | +49.99 | +3.1 | 0.860 |
| D..... | *A* | 83.16 | 83.16 | +33.16 | +0.960 | 4.060 |
| | *B* | 7.09 | 90.25 | +40.25 | +1.296 | 0.336 |
| | *C* | 4.08 | 94.33 | +44.33 | +1.583 | 0.287 |
| | *D* | 4.08 | 98.41 | +48.41 | +2.158 | 0.575 |
| | *E* | 1.60 | 100.01 | +50.01 | +3.1 | 0.942 |
| E..... | *A* | 62.59 | 62.59 | +12.59 | +0.321 | 3.421 |
| | *B* | 12.41 | 75.00 | +25.00 | +0.675 | 0.354 |
| | *C* | 8.22 | 83.22 | +33.22 | +0.963 | 0.288 |
| | *D* | 14.16 | 97.38 | +47.38 | +1.940 | 0.977 |
| | *E* | 2.62 | 100.00 | +50.00 | +3.1 | 1.160 |
| F..... | *A* | 63.28 | 63.28 | +13.28 | +0.339 | 3.439 |
| | *B* | 13.28 | 76.56 | +26.56 | +0.725 | 0.386 |
| | *C* | 7.07 | 83.63 | +33.63 | +0.979 | 0.254 |
| | *D* | 14.66 | 98.29 | +48.29 | +2.118 | 1.139 |
| | *E* | 1.72 | 100.01 | +50.01 | +3.1 | 0.982 |
| G..... | *A* | 43.22 | 43.22 | − 6.78 | −0.171 | 2.929 |
| | *B* | 13.57 | 56.79 | + 6.79 | +0.171 | 0.342 |
| | *C* | 7.54 | 64.33 | +14.33 | +0.367 | 0.196 |
| | *D* | 27.14 | 91.47 | +41.47 | +1.370 | 1.003 |
| | *E* | 8.54 | 100.01 | +50.01 | +3.1 | 1.730 |
| H..... | *A* | 4.46 | 4.46 | −45.54 | −1.700 | 1.400 |
| | *B* | 2.32 | 6.78 | −43.22 | −1.492 | 0.208 |
| | *C* | 1.78 | 8.56 | −41.44 | −1.364 | 0.128 |
| | *D* | 41.35 | 49.91 | − 0.09 | −0.002 | 1.362 |
| | *E* | 50.09 | 100.00 | +50.00 | +3.1 | 3.102 |

From the determinations made above it is clear that the eight populations taken from eight different levels of the body differ from each other. For the final estimate desired of the ranges of the three intermediate classes the ranges are weighted. For weighting, the percentage above or below the class in question, whichever is smaller, is multiplied by the value for the range determined in Table 8. A more accurate weighting could be obtained by multiplying also by the total number in each population, but since the populations are roughly of the same size this step is not included. The weighting and final weighted averages are shown in Table 9. The final estimate shows that the ranges of classes *B*, *C*, and *D* are in the proportion of 0.19:0.12:0.69 when expressed as frac-

TABLE 9

ESTIMATE OF RANGES OF CLASSES *B*, *C*, AND *D*; WEIGHTING OF ESTIMATES FOR THE EIGHT LEVELS

(*D. dorotocephala*)

| LEVEL | *B* | | *C* | | *D* | |
|---|---|---|---|---|---|---|
| | Weight | Range | Weight | Range | Weight | Range |
| A | 1.84 | 0.076 | 2.21 | 0.308 | 4.42 | 1.668 |
| B | 31.94 | 0.237 | 40.84 | 0.159 | 10.47 | 1.328 |
| C | 16.52 | 0.540 | 10.48 | 0.280 | 1.24 | 0.986 |
| D | 9.76 | 0.336 | 5.68 | 0.287 | 1.60 | 0.575 |
| E | 24.00 | 0.354 | 16.78 | 0.288 | 2.62 | 0.977 |
| F | 23.45 | 0.386 | 16.38 | 0.254 | 1.72 | 1.139 |
| G | 43.22 | 0.342 | 35.68 | 0.196 | 8.54 | 1.003 |
| H | 4.46 | 0.208 | 6.78 | 0.128 | 8.56 | 1.362 |
| Total | 155.19 | ..... | 134.83 | ..... | 39.17 | ..... |
| Weighted averages | ..... | 0.3426 | ..... | 0.2121 | ..... | 1.2295 |

Total range of classes *B*, *C*, and *D* = 1.7842
Proportions of total range occupied by classes *B*, *C*, and *D* = 0.19:0.12:0.69

tions of the total range of the three categories. It is of interest at this point to indicate the close agreement of this proportion to that of 0.18:0.13:0.69 arrived at from the estimate of ranges made by Method I.

Having arrived at an evaluation of the ranges of classes *B*, *C*, and *D*, it is now possible to locate the distribution of any of the eight populations relative to them. Each population is divided into three classes in such a fashion that the numbers in the classes are as nearly equal as possible. For the middle class the inverse probability function ($prf^{-1}[\Sigma p - 50]$) is found for the upper and lower limits (values already recorded in Table 8). The reciprocal of the difference between these values, multiplied by the theoretical class range, is the standard deviation. The mean of the population relative to the class limits is located by multiplying either the upper or the lower class limit, as ob-

tained above, by the standard deviation. For all possible middle classes used in making the application the following are the theoretical class limits:

| Middle Class | Theoretical Range |
|---|---|
| $B$ | 0.19 |
| $C$ | 0.12 |
| $B + C$ | 0.31 |
| $D$ | 0.69 |
| $C + D$ | 0.81 |
| $B + C + D$ | 1.00 |

The actual application of the method to the determination of the population means and standard deviations is shown in Table 10, A. Figures are here given for the eight levels studied as well as for the selected group studied in Method I.

A summary of the values for the means and standard deviations of the nine populations is shown in Table 10, B. The means are located from the values for the upper-class limits on a scale such that the lower threshold for class $B$ (anophthalmia) is at 0 and the upper limit for class $D$ (teratophthalmia) is at $+1$.

Method II provides a way for describing the variability of populations made up of assorted head types and for locating the means of the populations relative to the ranges of the three intermediate classes. Having described two populations (experimental and control groups, for instance) in this fashion, it is then possible to compare them. Examples will be given in the following section on the application of the method.

## APPLICATION OF METHODS TO THE EVALUATION OF HEAD-FREQUENCY DATA

In the following paragraphs and accompanying graphs and tables are presented examples of the application of different methods to the evaluation of existing head-frequency data. All the data used are taken from work on *D. dorotocephala*. For calculations on the basis of the older scale of equal intervals, values of 50, 40, 30, 20, 10 have been assigned to the head types from normal to acephalic. For calculations on the basis of values determined by Method I of this paper the values given in Table 4 have been rounded off to the nearest whole number so that the indices used have been as follows: normal, 70; teratophthalmic, 56; teratomorphic, 48; anophthalmic, 46; and acephalic, 35. It is assumed for the sake of illustration that the ranges of the different classes for the stocks used by other workers are the same as those of the stock used by the author. As will be pointed out later, there are sufficient reasons for believing that this assumption is not always a valid one, a fact to be borne in mind in considering the comparisons here made. In the sets of graphs intended for comparison the range 10–50 in the one case and 35–70 in the other have been allotted the same distance along the ordinate in order that the graphs may be directly compared.

Figures 4 and 5 are graphical presentations of data recorded by Child (1916, Table 2, p. 106). The ordinates in each figure represent head-frequency indices calculated for pieces from the body-levels indicated as abscissas; data for Figure 4 have been calculated on the older scale of values, and for Figure 5 on the values determined by Method I. Very little difference in the curves appears as a consequence of the change in the indices. The new scale does, however, accomplish for most levels of the body a greater separation of the ordinates for experiment (broken lines) and control (solid lines). This separation

brings out more clearly the effect of the experimental treatment—in this case the subjection of the experimental pieces to cyanide.

TABLE 10

LOCATION OF THE DISTRIBUTIONS OF SEPARATE POPULATIONS

(*D. dorotocephala*)

A. CALCULATION OF STANDARD DEVIATIONS AND LOCATION OF MEANS RELATIVE TO CLASS LIMITS

| | Limits | | |
|---|---|---|---|
| *A pieces:* | | | |
| *C* | −1.704 | −0.70 | |
| *D* | −0.036 | −0.01 | |
| *D* | 1.668 | 0.69 | |
| $\sigma = \frac{0.69}{1.668} = 0.41$ | | | |
| *B pieces:* | | | |
| *C* | −0.073 | −0.04 | |
| *D* | +1.255 | +0.65 | |
| *D* | 1.328 | 0.69 | |
| $\sigma = \frac{0.69}{1.328} = 0.52$ | | | |
| *C pieces:* | | | |
| *A* | +0.434 | +0.15 | |
| *B* | +0.974 | +0.34 | |
| *B* | 0.540 | 0.19 | |
| $\sigma = \frac{0.19}{0.54} = 0.35$ | | | |
| *D pieces:* | | | |
| *A* | +0.960 | +0.55 | |
| *B* | +1.296 | +0.74 | |
| *B* | 0.336 | 0.19 | |
| $\sigma = \frac{0.19}{0.336} = 0.57$ | | | |
| *E pieces:* | | | |
| *A* | +0.321 | +0.15 | |
| *C* | +0.963 | +0.46 | |
| *B*+*C* | 0.642 | 0.31 | |
| $\sigma = \frac{0.31}{0.642} = 0.48$ | | | |
| *F pieces:* | | | |
| *A* | +0.339 | +0.16 | |
| *C* | +0.979 | +0.47 | |
| *B*+*C* | 0.640 | 0.31 | |
| $\sigma = \frac{0.31}{0.64} = 0.48$ | | | |
| *G pieces:* | | | |
| *A* | −0.171 | −0.10 | |
| *C* | +0.367 | +0.21 | |
| *B*+*C* | 0.538 | 0.31 | |
| $\sigma = \frac{0.31}{0.538} = 0.58$ | | | |
| *H pieces:* | | | |
| *C* | −1.364 | −0.696 | |
| *D* | −0.002 | −0.001 | |
| *D* | 1.362 | 0.69 | |
| $\sigma = \frac{0.69}{1.362} = 0.51$ | | | |
| *Method I group:* | | | |
| *A* | −0.385 | −0.33 | |
| *D* | +0.791 | +0.67 | |
| *B*+*C*+*D* | 1.176 | 1.00 | |
| $\sigma = \frac{1.00}{1.176} = 0.85$ | | | |

B. SUMMARY AND LOCATION OF MEANS RELATIVE TO A SCALE ON WHICH THE LOWER LIMIT FOR CLASS *B* IS AT 0, AND THE UPPER LIMIT FOR CLASS *D* AT +1

| Group | Mean | Standard Deviation | Group | Mean | Standard Deviation |
|---|---|---|---|---|---|
| *A* pieces | +1.01 | 0.41 | *F* pieces | −0.16 | 0.48 |
| *B* pieces | +0.34 | 0.52 | *G* pieces | +0.10 | 0.58 |
| *C* pieces | −0.15 | 0.35 | *H* pieces | +1.00 | 0.51 |
| *D* pieces | −0.55 | 0.57 | Method I | +0.327 | 0.85 |
| *E* pieces | −0.15 | 0.48 | | | |

Data for the graphs shown in Figures 6 and 7 have been taken from Buchanan (1923, Table 2, p. 407). Ordinates for Figure 6 have been calculated by the older scale of indices and for Figure 7 by the indices of Method I. The curves for experimental (broken line)

and control pieces (solid line) show little change in their general form or in their position relative to each other as a result of the change in indices.

Data for the graphs shown in Figures 8 and 9 are taken from Hinrichs (1924, Table 5, p. 283). Figure 8 is on the basis of the older head-frequency indices, and Figure 9 on the basis of those of Method I. Attention here is directed to the effect of the experimental treatment (caffein) on the head frequency of pieces at the B level. From Figure 8 it appears that the head frequency of pieces in the experimental group (broken line) is higher than that of the controls (solid line), but in Figure 9 the relationship is reversed.

TABLE 11

EVALUATION OF DATA FROM HINRICHS' EXPERIMENT

(*D. dorotocephala*)

A. COMPARISON OF MEANS DETERMINED BY USE OF HEAD-FREQUENCY INDICES

| | A | | | B | | | C | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | $m_e - m_c$ | $P$* | Mean | $m_e - m_c$ | $P$ | Mean | $m_e - m_c$ | $P$ |
| Indices of equal-interval scale: | | | | | | | | | |
| Experimental | 45.60 | +1.6 | 0.0455 | 26.40 | +0.80 | 0.500+ | 19.20 | −2.20 | 0.230 |
| Control | 45.00 | | | 25.60 | | | 21.40 | | |
| Indices of Method I: | | | | | | | | | |
| Experimental | 64.20 | +1.08 | 0.2713 | 46.50 | −0.32 | 0.500+ | 41.69 | −1.95 | 0.109 |
| Control | 63.12 | | | 46.82 | | | 43.64 | | |

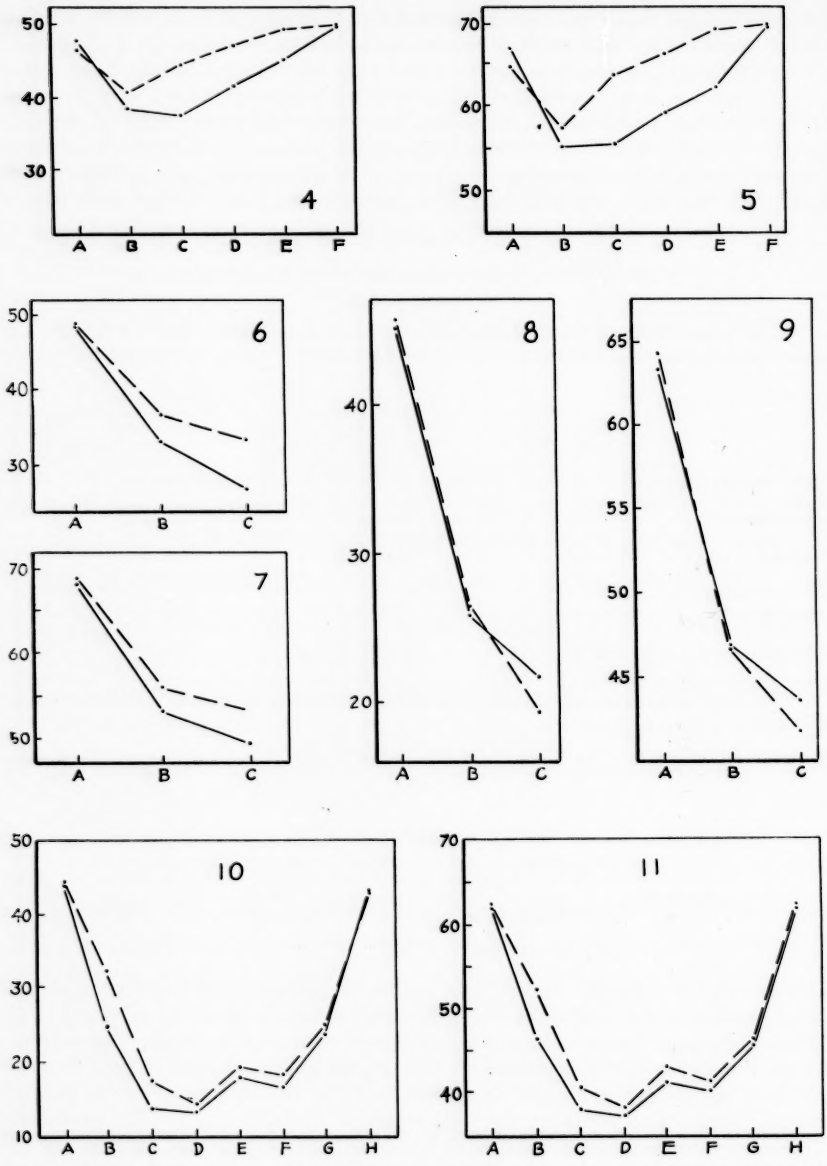
* $P$ = probability of chance occurrence of difference as determined by method for standard error of the difference between means.

B. DESCRIPTION OF GROUPS BY METHOD II

| | A | | B | | C | |
|---|---|---|---|---|---|---|
| | Mean | $\sigma$ | Mean | $\sigma$ | Mean | $\sigma$ |
| Experimental | +1.12 | 0.49 | +0.15 | 0.48 | −0.13 | 0.39 |
| Control | +1.00 | 0.38 | +0.15 | 0.36 | −0.02 | 0.47 |

In either case the difference between the indices for experimental and control groups is so slight that it is not likely that any significance would be attached to it.

These data from Hinrichs have been subjected to further analysis. The differences between the means of the experimental and control groups at each of the three levels have been tested for significance by the test for the standard error of the difference between means. The pertinent data are recorded in Table 11, A. The probabilities of chance occurrence of such differences listed indicate that, on the basis of the older indices, the difference at the A level is possibly significant but that no significance can be attached to the differences between the experimental and control groups at levels




FIGS. 4 and 5.—Graphical presentation of data from Child (see text) on the effect of cyanide on head frequency of one-sixth pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken

B and C. Comparison between the means determined by the use of the indices of Method I of this paper indicates that none of the differences are significant, except that at the C level the difference ($P = 0.109$) may be suggestive of a trend.

The procedure developed by Wright (1934) and outlined in Method II of this paper has been applied to Hinrich's data, and the ranges of the three middle classes have been determined as in the proportion of 0.12:0.10:0.78. Using these values for the theoretical class ranges, the means and their theoretical standard deviations have been calculated relative to a scale on which the threshold for anophthalmia is at 0 and for normal at +1. This information is tabulated in Table 11, B. While this procedure locates the means relative to each other and to the ranges of the three middle classes and gives a measure of the variability of the groups, it does not afford any reliable way of making further comparisons or statistical evaluations. The standard errors based on the derived standard deviations do not constitute an adequate basis for a statistical test. The method gives a description of the groups but no more. The values determined indicate for levels A and C the same relationship between experimental and control groups as that revealed above by the use of either scale of head indices, a relationship shown graphically in Figures 8 and 9. At level B the calculated means fall at the same point on the scale.

Hinrichs' conclusion in regard to this and similar experiments, that as a result of the experimental treatment "head frequency is lowered in C pieces and slightly raised in A pieces," has been made on the basis of the percentage distribution of head types without resort to any statistical treatment of the data. The analysis of the single experiment given above can be taken to indicate only that the single experiment is not conclusive but that the trends are in the direction Hinrichs has indicated. If the same trends are present in her other experiments as in the one studied here, it is probable that more complete analysis would give statistical evidence to substantiate her conclusion.

The curves presented in Figures 10 and 11 are based on unpublished data on the effect of aggregation on head formation in *D. dorotocephala*. The curves of Figure 10 are computed from the values of the equal-interval, head-frequency scale, and those of Figure 11 on the basis of the indices determined by Method I. The points along the

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lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 4 are calculated on the basis of the equal-interval scale, those for Figure 5 on the basis of the indices determined by Method I.

FIGS. 6 and 7.—Graphical presentation of data from Buchanan (see text) on the effect of chloretone on head frequency of one-third pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 6 are calculated on the basis of the equal-interval scale, those for Figure 7 on the basis of the indices determined by Method I.

FIGS. 8 and 9.—Graphical presentation of data from Hinrichs (see text) on the effect of caffein on head frequency of one-third pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 8 are calculated on the basis of the equal-interval scale, those for Figure 9 on the basis of the indices determined by Method I.

FIGS. 10 and 11.—Graphical presentation of data from the author's experiments on the effect of aggregation on head frequency of one-eighth pieces of *D. dorotocephala*. Ordinates represent head-frequency indices, and abscissas the levels of the body. Points along the broken line are averages for 10 experiments with pieces isolated in 5 ml.; points along the solid line are averages for the 10 paired experiments with pieces in groups of 10 in 50 ml. Ordinates for Figure 10 are calculated on the basis of the equal-interval scale, those for Figure 11 on the basis of the indices determined by Method I.

broken lines represent averages of the results of 10 separate experiments, each experiment consisting of 10 fragments from each of the 8 levels of the body indicated as abscissas, each fragment isolated in 5 ml. of water. Each of these experiments was

TABLE 12

EVALUATION OF DATA FROM AGGREGATION EXPERIMENTS DISCUSSED IN TEXT AND PRESENTED IN FIGURES 10 AND 11

(*D. dorotocephala*) (Ten paired experiments)

A. COMPARISON OF VALUES FOR MEANS OF ISOLATED AND GROUPED PIECES DETERMINED BY THE USE OF HEAD-FREQUENCY INDICES

| | A | B | C | D | E | F | G | H | Entire Series |
|---|---|---|---|---|---|---|---|---|---|
| Indices of equal-interval scale: | | | | | | | | | |
| Isolated mean | 44.79 | 32.78 | 17.40 | 14.19 | 20.46 | 18.16 | 25.24 | 43.28 | ..... |
| Group mean | 44.11 | 25.08 | 13.74 | 13.21 | 18.07 | 16.62 | 24.23 | 43.56 | ..... |
| $m_I - m_G$ | 0.68 | 7.70 | 3.66 | 0.98 | 2.39 | 1.54 | 1.01 | − 0.28 | ..... |
| $P$* | 0.269 | 0.0014 | 0.034 | 0.563 | 0.199 | 0.392 | 0.698 | 0.847 | 0.041 |
| Indices of Method I: | | | | | | | | | |
| Isolated mean | 62.78 | 52.28 | 40.70 | 38.43 | 43.14 | 41.46 | 46.78 | 62.92 | ..... |
| Group mean | 62.36 | 46.59 | 38.09 | 37.41 | 41.38 | 40.32 | 45.85 | 62.35 | ..... |
| $m_I - m_G$ | 0.42 | 5.69 | 2.61 | 1.02 | 1.76 | 1.14 | 0.93 | 0.57 | ..... |
| $P$ | 0.632 | 0.0058 | 0.040 | 0.444 | 0.199 | 0.444 | 0.629 | 0.701 | 0.023 |

* $P$ = probability of chance occurrence of differences as determined by "Student's" method.

B. VALUES FOR MEANS AND STANDARD DEVIATIONS OF ISOLATED AND GROUPED PIECES DETERMINED BY METHOD II

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| Isolated mean | +0.98 | +0.49 | −0.14 | −0.61 | 0.00 | −0.12 | +0.16 | +1.02 |
| Isolated $\sigma$ | 0.30 | 0.50 | 0.40 | 0.67 | 0.39 | 0.44 | 0.56 | 0.47 |
| Grouped mean | +0.95 | +0.13 | −0.26 | −1.20 | −0.28 | −0.20 | +0.09 | +1.05 |
| Grouped $\sigma$ | 0.40 | 0.74 | 0.36 | 1.10 | 0.68 | 0.48 | 0.66 | 0.52 |
| $m_I - m_G$ | +0.03 | +0.36 | +0.12 | +0.59 | +0.28 | +0.08 | +0.07 | −0.03 |

Comparison of means of entire series by "Student's" method

$P$ = 0.035

paired with an experiment set up at the same time in which the 10 fragments from each level were grouped in 50 ml. of water. The averages for these 10 experiments with grouped pieces are indicated by the points along the solid lines.[2]

[2] The result tabulated here appears to indicate that in this particular series of experiments and with the volumes used the isolated pieces formed better heads than grouped pieces. This conclusion is not to be taken as representative of all the experiments, for in other combinations of volumes the grouped pieces were at times favored.

The choice of the scale of indices makes little apparent difference in the relationship revealed between the isolated pieces and the grouped pieces, as is indicated by comparing Figures 10 and 11. The indices of Method I bring about a reversal of the points at level H and bring the trend at that level into conformity with the trends at each of the other seven levels of the body. Since the points represent averages for paired experiments, it is possible by the use of "Student's" method ("Student," 1925) to determine the significance of the differences indicated. The *P* values for these tests are shown in Table 12, A. With either scale of indices it appears that the differences at levels B and C are probably significant. At levels A, D, E, F, and G the isolated pieces are favored, but in no case is the difference a significant one. Since the differences at level H are of no significance, the reversal of the relationship between isolated and grouped pieces is of no consequence, although the situation indicated by the use of the indices of Method I seems more plausible since the isolated pieces are favored at the other 7 levels. Confidence is felt in the application of the indices of Method I to these particular data, since the indices are developed on the basis of the observed frequency distribution of head types in these experiments and in others performed on worms from the same stock.

In a case like this one it is perhaps helpful to have some test to express the result of the whole series of experiments rather than the separate effect at each of the eight levels. By comparing the means of isolated and grouped pieces at each of the eight levels, it is possible now to test the significance of the entire series by regarding the means of the values for isolated and grouped pieces at each level as a pair of values and testing the series of 8 pairs by "Student's" method. The *P* values for these comparisons are shown in the final column in Table 12, A. They indicate that, when the results are calculated by either of the sets of indices used, the isolated pieces have formed heads which are probably significantly better than those formed by grouped pieces.

For this same series of experiments on isolated and grouped pieces Method II has been used to determine the means and standard deviations of the sixteen separate populations relative to the ranges of the three intermediate classes of head types. This information is tabulated in Table 12, B. It shows for the levels A–G, inclusive, that each mean for isolated fragments lies above the mean for its respective control of grouped pieces. At level H the mean for the grouped pieces is slightly above that for the isolated pieces, a difference also indicated when the results are calculated by the older (10–50) scale of indices. The average of the differences between the means (comparing at each level the mean for isolated pieces with the mean for grouped pieces) is +0.15. The probability that such a difference is due to chance is 0.035 ("Student's" method). This confirms the conclusion arrived at by assigning indices, i.e., that isolated pieces have formed better heads than grouped pieces in this particular set of experiments.

It would be possible to make further comparisons of the data from these experiments by throwing together the result of all 10 experiments with isolated pieces and comparing it with the total of the experiments on grouped pieces. Comparisons could then be made for the eight levels by use of the method for the standard error of the difference between means. There is nothing to be gained by such a comparison. "Student's" method has a distinct advantage for the analysis, since it takes into account the pairing of the experiments and since each experiment is compared directly with a control experiment set up at the same time.

In general, any individual curve drawn by use of the indices developed in Method I

is little different from one drawn by use of the older indices. This has been sufficiently shown in the cases already discussed and illustrated. When applied to isolated experiments, in which small numbers of individuals have been used, a reversal of the slope of a small portion of the curve may be effected. An example is given in Figure 12, the result of a single experiment with 10 worms (10 pieces at each of the eight levels). The solid line represents a plotting of the head-frequency data on the basis of the indices of the 10–50 scale, and the broken line the same data computed on the basis of the 35–70 scale of Method I. The slope of the curve between levels F and G is reversed by the change in the scale of indices. Such a reversal consistently occurring might demand

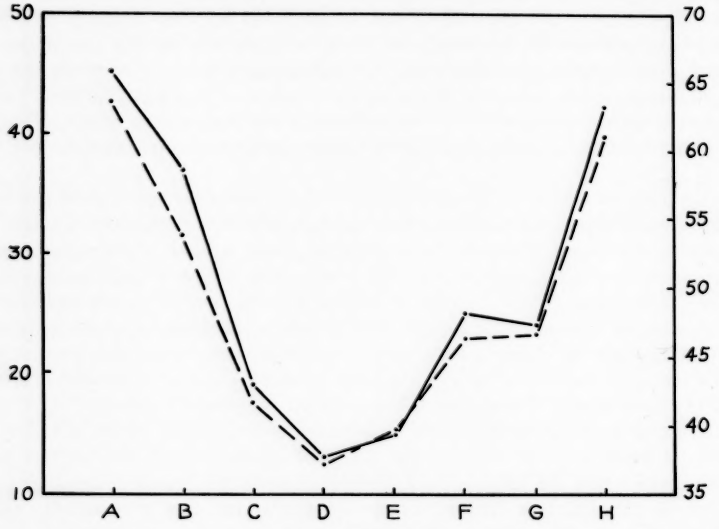


FIG. 12.—Graphical presentation of data from a single experiment with *D. dorotocephala;* one-eighth pieces, 10 from each level, each piece isolated in 1 ml. of water. The levels of the body are indicated as abscissas. Ordinates for the points along the solid line are calculated on the basis of the equal-interval scale (on left) and for the points alone the broken line on the basis of the indices are determined by Method I (on right).

a reinterpretation of head-frequency phenomena as related to the axis of the worm. It appears, however, that the reversal is the result of the small number of cases and of peculiarities of the particular data used. In averages of experiments or in experiments where considerable numbers of pieces are involved, such changes in slope have not been observed to occur. In general, a change to the revised scale of indices results in a "smoothing" of the curve but in no great change in its general character.

## DISCUSSION

The methods presented in the preceding pages represent an attempt at more accurate expression and evaluation of head-frequency data from experiments with planarians. By the procedure outlined in Method I definite values are assigned to the different

types of heads and are based upon the observed frequency of occurrence of those types. Its application depends upon the availability of data from experiments in which the experimental groups are small, in order that many units may be selected, each of which shows the whole range of head types. It appears appropriate to apply such a method to the evaluation of data from regeneration experiments in which small numbers of pieces must of necessity be used. When each of these experiments is paired with an experiment set up at the same time and the paired experiments are repeated a number of times, the significance of the values determined may then be tested by a statistical method such as that presented by "Student" (1925).

Method II of this paper, developed by Wright, is one more suitable for expressing the results of experiments in which large numbers of pieces have been used and the experiments have not been replicated. It depends for its application upon the determination of the ranges for the three middle classes of the head-type scale. The variability of the regenerated structures can then be described, and the mean of the group located with respect to the ranges of the three middle categories.

Sufficient evidence has been presented to demonstrate that the head indices of Method I and the class ranges of Method II are not constants to be used in all work with a particular species. Wright determined from the combined data of three separate workers that the ranges of the three intermediate head classes were in the proportion of 0.36:0.14:0.50. The application of Method II to Hinrichs' data in a preceding section revealed that in her experiments the ranges of the same three categories were in a proportion of 0.12:0.10:0.78. The ranges determined by the application of this method to the present author's data are in the proportion of 0.19:0.12:0.69. This proportion is almost identical to that arrived at by Method I from a study of selected experimental units showing the whole range of head types (0.18:0.13:0.69). The similarity of the two proportions last cited, determined by different methods from the same data, confirms the adequacy of Method I for the determination of these ranges.

No attempt will be made to reconcile the differences in distribution of head types brought out above. Differences in the duration of the experiments considered or differences in the classification of regenerated heads may be sufficient to account for a considerable discrepancy. This more or less subjective error may be introduced at either end of the head-frequency scale. Disagreement between workers on the degree of disparity in size of the eyes necessary to cause a head to be rated as teratophthalmic rather than normal would account for a shift in the threshold between normal and teratophthalmia. A shift at this end of the scale may be caused by experiments of different length, for it has been observed by some workers that, if teratophthalmic pieces are followed over a period of time, some regulation of the eye size may take place, and eyes once classified as unequal in size may later be considered as of normal proportions. At the lower end of the scale, workers may disagree in their evaluation of the amount of regenerated tissue necessary to constitute an anophthalmic outgrowth, or, if allowed to reconstitute over a period of time, an appreciable number of pieces once classified as acephalic will develop anophthalmic outgrowths. Either of these events will cause an apparent shift in the position of the threshold between acephaly and anophthalmia. It is, in addition, probable that the stocks used by different workers differ not only in their head-forming ability (Rulon, 1938, p. 210) but also in the ranges of the head classes along the head-frequency scale. These considerations make it imperative that application of either of the new methods of evaluation presented in this paper should

not be undertaken unless the ranges of the classes of heads are first determined for the particular stock of worms being used. Unless this is done, neither scheme appears to have any distinct advantage over the scale of indices that has long been used.

There is no available measure of the success of planarian regeneration other than the classification of the head types into visually recognizable categories. The differences brought out between different stocks and the observations of different workers in classifying head types do not imply that this classification is an unreliable method for the evaluation of head frequency. The differences do, however, indicate that caution must be exercised in comparing the observations of different workers and of the same or different workers when the observations are based on experiments in which the duration of the period of regeneration is not constant or worms from different stocks are used.

It is assumed that for a given stock the experimental treatment will not greatly modify the values for the indices or for the ranges of the head classes. This assumption may not be strictly true if the experimental treatment is severe and the differences in head frequency between experimental and control groups are great. It is necessary, however, to proceed on such an assumption in order to have a common basis for comparison. If the differences are so great that they cause great changes in the indices or class ranges, it is likely that the result of the experiment will be sufficiently clear without the necessity for more complete analysis of the data.

Exception may be taken to the choice of the class medians as indices in Method I of this paper. If the populations being compared differ greatly from each other, the positions of the medians will be inconstant. The values for the intermediate groups will remain fairly constant relative to each other, so long as these groups are located near the mean for the entire population being considered. Median values for the extreme classes (normal and acephalic) will be most inconstant if the numbers of individuals in the extreme classes are small and if the two populations differ greatly in their central tendency. For use in comparing results from experiments in which the populations differ greatly in their distribution the mid-point of the range for each class might be more representative. As long as the intermediate classes are small and near the mean of the entire range, the values for the mid-points of those classes will not differ greatly from those for their medians. For the two extreme classes the medians and mid-points will be farther separated. The choice in this case is purely arbitrary. Since the indices are intended for strict application only in experiments on relatively homogeneous stocks for which the indices have been determined and since the extreme classes are both large, it is thought that no serious error will be introduced by the use of the medians. The essential feature is not the choice of the value but rather its consistent application. The use of these definite values for the head types possesses a real advantage in the expression of experimental results, and, as has been pointed out, these indices will be more critical than the arbitrarily assigned values long used if they are determined on the basis of the observed frequencies of occurrence of the head types.

No account has been taken of the factors involved in the determination of the type of head. These factors may be intrinsic or extrinsic and have been rather fully analyzed in the papers of Child and of his students (Child, 1912, 1914, 1916, 1920; Child and Watanabe, 1935; Buchanan, 1922, 1923; Rulon, 1936, 1937; etc.). Given carefully controlled external conditions to which the fragments are subjected, some variation in the type of head formed is to be expected as a consequence of inequalities in the size of

fragments or differences in the physiologic condition of the individual worms from which the fragments have been isolated. Such differences are sufficient to account for the formation of heads of all types in small groups of pieces. Similarly, minute and uncontrollable differences in the conditions in the immediate vicinity of fragments placed even in the same container may be sufficient to account for variations in the types of heads regenerated. Indeed, without variations due to one or the other of these types of factors such an analysis as is here attempted could not be made. In the experiments used as the basis for these observations it seems most likely that the primary causes for lack of uniformity within the units of 10 pieces may be those traceable to differences in size of piece or physiologic condition of the individual worms. These differences are impossible to control, even though all pieces are cut by the same person and the 10 worms used in each experiment are from the same source and have had identical histories of laboratory culture. Regardless of the cause of the variation observed, the method of analysis would remain the same.

The differences observed in the calculated values of Method I to be used in estimating the head frequency in the two species of *Dugesia* here considered are perhaps to be anticipated. There is no a priori reason for expecting that the distribution of head types will be the same for all species. Indeed, the estimations of class ranges already made for different data from *D. dorotocephala* are indicative of differences within a species. Such a difference also has been observed in the author's experience with *D. tigrina*. The data already presented for this species are based on experiments with worms from well-known collecting grounds in the town of Falmouth, Massachusetts, and from a stream near East Providence, Rhode Island. Worms from these localities appear to be similar in life-history and behave similarly in regeneration. A few experiments with worms collected from Mary's Lake on Naushon Island near Woods Hole, Massachusetts, have yielded data which indicate that they behave differently in regeneration. As has been pointed out by Curtis (1902) and Kenk (1937), worms collected from this locality have never been observed to pass through a sexual phase in their life-history. Because of this failure to exhibit reproductive organs, their identification remains uncertain, although they are believed to constitute a race or variety of *D. tigrina*. The distribution of head types observed in this race is very different from that recorded in Table 2. It indicates that in this species as in *D. dorotocephala* no one scale of indices or estimate of class ranges is satisfactory for application to all data obtained from work done with the particular species.

It is not to be inferred that the originators and users of the old scale of indices intended it to be more than an approximation for convenience. Indeed, Buchanan has stated (1923, p. 410) that "no claim is made that the values 5, 4, 3, 2, 1, represent any exact mathematical valuation of regenerated tissue; this is simply a convenient and consistent way of showing the effects of the agent on head frequency." For the purpose designed, the old scale of equal intervals appears in most cases to have been entirely adequate for outlining major differences in head frequency along the axis of the planarian body or for distinguishing between the head frequencies of experimental and control groups where the differences are not obscure. Where such differences are slight, however, the use of a scale in which the indices or class ranges do closely approach the values based on observed frequencies of occurrence of the regenerated structures probably comes closer to revealing the real relationships between control and experimental groups.

SUMMARY

1. Methods are presented for the determination of the class ranges, limits, and medians for the different types of anterior regenerated structures in planarians. These values are based upon the observed frequencies of occurrence of the five recognizable head types.

2. The chosen indices of Method I are the abscissas of the medians of the different classes, located relative to a scale of 0–100 embracing the entire range of variability of head types. The determination of these indices is made on the basis of the percentage distribution of head types in many small, experimental units, each unit showing the whole range of the classes of head types. The indices appear most suitable for use in the evaluation of data from experiments in which the numbers of pieces in each experiment are of necessity small.

3. By Method II, an application of a method developed by Wright, the theoretical ranges of the three intermediate classes of heads are determined by statistical means from data from experiments with large numbers of pieces from a homogeneous stock. The mean for any particular population of regenerated structures may then be located relative to the ranges of the three intermediate classes and its variability described in terms of a derived standard deviation. Application of the method may be made to experiments in which the indices of Method I could not be determined from the available data.

4. Cases chosen for illustration demonstrate the application of different available methods to the evaluation of head-frequency data. Use of the revised indices of Method I may bring about an apparent reversal of the relationship between experimental and control groups, as compared to the same data evaluated by the indices of the equal-interval scale of previous workers. The availability of several methods makes possible the use of more than one method for the evaluation of data. When followed by a statistical test, a comparison of the results of application of more than one method will confirm the significance of experiments the outcome of which is in doubt.

5. Differences brought out in the distribution of head types formed by different species or by planarians from different stocks within a species or as determined by different workers make it essential that any indices or estimates of ranges intended for strict application must be determined for the particular body of data being evaluated.

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# A MIGRATION-DILUTION APPARATUS FOR THE STERILIZATION OF PROTOZOA[1]

(One plate and one figure)

C. LLOYD CLAFF
Arnold Biological Laboratory, Brown University, and Marine Biological Laboratory, Woods Hole

## I. INTRODUCTION

PROTOZOA may be divided roughly into large groups depending upon how they respond to various influences. One such large group is negatively geotropic. The apparatus described herein has been designed to separate this type of protozoa from their associated bacteria, provided the protozoa will behave in a negatively geotropic manner in a closed vessel.

Many different techniques for the sterilization of protozoa have been employed in the past, and, while it has been demonstrated that it is possible to obtain sterile organisms by these methods, they all have very serious limitations. Hargitt and Fray (1917) and Parpart (1928) used methods employing a dilution factor as the predominant means for effecting sterilization by washing in Petri dishes. Cleveland (1928) made use of lateral migration causing protozoa to migrate across a 10-inch Petri dish. Hetherington (1934) combined both migration and dilution in a series of washes and lateral migrations in depression slides inclosed in Petri dishes. The chance for contamination due to manipulations inherent in these methods is considerable, because large areas of fluid surface are exposed to air contamination every time an organism is transferred into or out of these dishes. Moreover, a considerable amount of time and work is necessary to sterilize one organism. Glaser and Coria (1930) caused organisms to migrate up a tube, but here the dilution factor is usually not great enough to take care of all the associated bacteria, particularly if by chance *Pseudomonas* or other rapidly moving bacteria are present in the culture.

The centrifuge method (Kidder and Stuart, 1939), employing at least fifteen washes, is limited in its application to situations where the investigator has a high concentration of protozoa to start with. The chances of contamination through faulty manipulative technique are extremely great in this method, involving as it does at least fifteen transfers from one sterile centrifuge tube to another by means of sterile pipettes.

In an attempt to overcome these limitations an apparatus was designed incorporating both a migration and a dilution factor in a closed system. The organisms are introduced at one end of the system and are not exposed to any chance contamination until they are taken away from the apparatus after the final migration. The dilution factor is of the order of $10^6$, and the amount of material recovered in a sterile condition is as great as or greater than any method heretofore described. The time necessary for the protozoa to complete the migration through the apparatus varies roughly from 20 minutes to 1 hour depending on the type of protozoa.

[1] I wish to acknowledge the timely suggestions of Professor G. W. Kidder. His constructive criticisms and his painstaking tests of this apparatus have aided greatly in its development.

## II. APPARATUS AND METHOD

The apparatus consists of a series of six flasks,[2] 8 inches tall, each holding approximately 145 ml. of fluid, joined together so that the top of one flask is connected by small-bore rubber tubing to the bottom of the next succeeding flask (Fig. 1). The rubber tubing is securely fastened to the glassware. A reservoir (capacity 1 liter) capable of being raised to a level higher than the flasks is joined to the bottom of flask No. 1 by rubber tubing. A test tube into which a glass tube is inserted through a cotton plug is connected by rubber tubing to the top of flask No. 6. The fluid in the raised reservoir is held in check by a spring clamp. By releasing the clamp it is possible to transfer 1.5 ml. of fluid from the reservoir into the bottom of flask No. 1. This effects a transfer of the uppermost 1.5 ml. of fluid from each flask into the bottom of the next succeeding flask in line. The uppermost 1.5 ml. of fluid in flask No. 6 is at the same time transferred into the test tube. A vaccine port is blown into the side of flask No. 1 near the bottom (Fig. 1), to which is fitted a vaccine cap. Through this cap the protozoa to be sterilized are injected with a sterile hypodermic needle.

The migration flasks and reservoir are mounted on a brass plate by means of cast-bronze rings held by brass posts (Pl. I). The cast-bronze rings are held against the glassware by light coiled springs which allow the glassware to expand during autoclaving.

The reservoir is assembled in a brass cradle capable of being raised and lowered on a brass rod firmly attached to the brass plate. It is lowered so that the entire apparatus will fit into the 10-inch door of a small autoclave. The brass plate, with the assembled flasks and reservoir, rests on, but is separable from, a wooden base 2 inches high which carries a suitable clamp for holding the final sterile test tube.

The apparatus is partially filled with fluid and sterilized. This fluid may be slightly alkaline distilled water or any other nonnutritive or nutritive media.

With the reservoir in the lowered position the fluid is adjusted in the flasks by means of an aspirator until flask No. 6 is empty and there is about 1 inch of fluid in flask No. 5 and the reservoir.

An auxiliary 1,000-ml. flask is three-quarters filled with the same fluid that is to be used in the migration apparatus, cotton plugged, and autoclaved with the apparatus, together with the following:

12 empty, cotton-plugged test tubes
1 No. 18 hypodermic needle in cotton-plugged serological tube
1 large 50-ml., cotton-plugged centrifuge tube
1 rack of micropipettes fitted with cotton plugs, each in its own serological tube
50 test tubes each filled with 2 ml. of some suitable fluid medium in which to culture the sterile organisms obtained

All these are autoclaved at 15 pounds' pressure for 20 minutes. This should be done at least 12 hours before the apparatus is to be used, in order to allow it to cool to room temperature and establish equilibrium to avoid convection currents. A 5-ml. glass syringe to be used for the injection of the protozoa to be sterilized is boiled for 20 minutes.

Organisms are sterilized as follows: The apparatus is placed on the wooden base, and the test tube is placed in the clamp provided for it so that it rests at table level below the level of the migration flasks. The wire clamp is tightened on the rubber tubing connecting the reservoir to flask No. 1. The reservoir is raised to the higher level and is then filled

[2] These may be obtained from Charles Kaziun, 338 Winter Street, Brockton, Massachusetts.

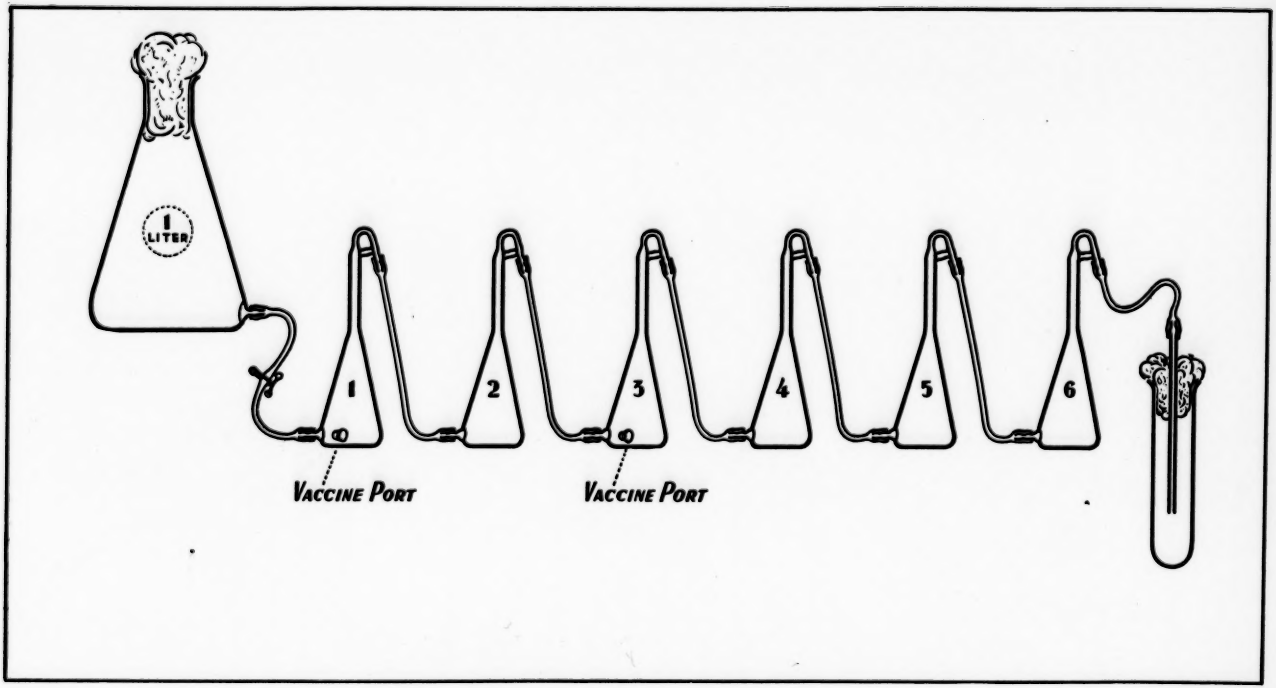


FIG. 1.—Diagram of migration-dilution apparatus drawn to show connections

with fluid from the auxiliary flask by removing both cotton plugs, flaming the necks, and pouring the liquid quickly from the auxiliary vessel into the reservoir, while holding a Bunsen burner so that the flame protects the point of operation from air contamination. The reservoir is again cotton plugged. The spring clamp is loosened, and the fluid is allowed to flow down into the migration flasks until they are completely filled, together with the tubing coming from flask No. 6. This rids the system of any air pockets. About 3 ml. of fluid are allowed to run into the test tube. The flow of liquid is now checked, and a dry sterile test tube is substituted for the tube containing the 3 ml. of fluid. The sterile dry cotton plug taken from the dry test tube is used to plug the tube taken from the apparatus, and the cotton plug, through which the glass "take-off" tube is passed, is used, of course, to plug the dry tube.

The protozoa to be sterilized are concentrated by centrifugation and, if the contamination is heavy, may be washed a few times by this method (Kidder and Stuart, 1939). This removes the debris and larger clumps of bacteria. The supernatant fluid (i.e., all but 2 ml.) is removed with a sterile 10-ml. pipette, and the cotton plug is replaced. A fresh sterile 10-ml. pipette is used to transfer the organisms in the remaining 2 ml. of fluid (less the very tip fluid) to the 50-ml. centrifuge tube. The protozoa are transferred from the 50-ml. centrifuge tube through the vaccine port to the bottom of flask No. 1 by a sterile hypodermic needle and syringe. The protozoa start to migrate up flask No. 1 (migration takes from 1 to 8 min. depending on the organisms used and their physiological state at the moment). The migration may be followed with a 5×–10× hand lens.

When sufficient numbers have accumulated at the top of flask No. 1, the spring clamp is released, and about 1.5 ml. of fluid is allowed to flow through. This may be gauged by the amount which flows into the test tube from flask No. 6. This operation deposits the protozoa in the bottom of flask No. 2, and they start to migrate to the top of this flask. The test tube is now replaced with a sterile dry one. When the protozoa are in sufficient numbers at the top of the second flask, they are transferred as above to the third flask. The first five test tubes taken off in this manner serve as controls for the sterility of the apparatus, inasmuch as the fluid from each may be poured on agar plates. We have never found a contaminated plate from this source.

The sixth and following test tubes taken off should contain the protozoa; and 0.5 ml. of the contents are taken off with a sterile pipette and deposited in a sterile Petri dish placed on the stage of a dissecting microscope, fitted with a Plastacele (Dupont) drape. The field is immediately searched, and single organisms are transferred by means of small sterile micropipettes to shell vials or test tubes containing either sterile nutrient media or sterile distilled water. If the protozoa are obligate bacteria-feeders, these vials have been previously bacterized with a light suspension of a pure strain of a favorable bacteria.

The following precautions are necessary for the successful operation of the apparatus.

Flask No. 6 should be empty, and there should be about 1 inch of fluid in flask No. 5 when the apparatus is autoclaved. This allows for expansion of fluid during autoclaving. If more fluid than this is allowed to remain in the system, it will flow over into the test tube, fill it, and soak the cotton plug.

The apparatus should be autoclaved without the spring clamp on the rubber tubing coming from the reservoir. Failure to do this will result in a blown-out rubber tube.

After the reservoir has been filled from the auxiliary flask, enough fluid should be allowed to flow through the apparatus to fill it completely, including the tube coming from the top of flask No. 6, thereby expelling all air bubbles from the system.

When injecting flask No. 1 with the protozoa, care should be exercised so that no air is introduced into the system with the protozoa. This is of extreme importance. Should an air bubble be introduced, it would rise rapidly in the flask and might destroy the sterility of the fluid therein and would travel ahead of the migration of the protozoa throughout the system carrying possible contamination with it.

The transfer of the glass take-off tube from one sterile test tube to another should be done with aseptic technique, being careful not to allow the glass tubing to touch the sides of the test tube.

Bacteria-feeding forms which encyst readily (*Tillina*, *Colpoda*, etc.) may be recovered in a pure bacteria-protozoa culture by the following modification:

Flask No. 3 is provided with a vaccine cap. The cap is covered with cotton and soaked with 70 per cent alcohol for at least 20 minutes before injection is made. As soon as the protozoa have completed the migration through the second flask and have been carried over into the bottom of the third flask, the fluid of this flask is supplied with a suspension of the food bacteria, injected through the vaccine cap.

The subsequent technique is as already outlined.

Flasks No. 1 and No. 6 may each be provided with an additional vaccine cap through which a platinum wire is inserted. A bead of glass bonded to the wire on the inside of the vaccine cap keeps the wire from being pushed out by pressure during autoclaving. These wires provide the means for passing an electric current through the fluid in the apparatus during migration, if the investigator wishes to study the effects of electric current as an aid to migration.

### III. EXPERIMENTAL EVIDENCE AND QUANTITATIVE BACTERIOLOGICAL TESTS FOR STERILITY

*Paramecium caudatum* were isolated from a wild environment and fed for 8 days through 2 transplants on live yeast. The culture was concentrated and put through the apparatus. Migration was very slow, but one *Paramecium* was recovered and placed in a tube of proteose-peptone broth. This tube was incubated for 3 weeks at 26° C. The broth remained clear and at the end of this period was flooded onto agar plates, and the plates remained entirely free of bacterial growth.

*Tillina canalifera*[3] were isolated from a hay infusion and grown on *Vibrio* sp. in association with other bacterial contaminants. One milliliter of this culture, concentrated by centrifugation, was injected into the apparatus. Very rapid migration took place. The entire migration consumed only 25 minutes. From 50 to 100 ciliates were recovered in tube No. 6, and about 50 organisms each in tubes Nos. 7, 8, 9, and 10.

Single organisms were not isolated, but the whole tube was bacterized with *Vibrio* sp. After considerable growth (48 hr.) 9 agar plates were streaked from tube No. 6, and 3 plates each were streaked from tubes Nos. 7, 8, 9, and 10.

Tube No. 6 showed only typical *Vibrio* sp. after a 10-day incubation period. Therefore, this is considered an adequate method for obtaining this ciliate in a pure bacteria-protozoa culture. Small numbers of extraneous bacteria appeared on the plates from tubes Nos. 7, 8, 9, and 10, however, and these tubes were discarded.

Many migrations of *T. canalifera* were made, and it was observed that the success of the migration was dependent on their physiological condition. If too well fed, they did

[3] This strain of *Tillina* was sent to Professor Kidder in 1937 through the courtesy of Dr. J. P. Turner and has been maintained in this laboratory ever since.

not migrate but formed division cysts on the sides of the flasks; if starved, they formed permanent cysts. Migration results were the best when the organisms used were within 4 or 5 hours of the time they normally would form protective cysts. They appeared to be most active at this period.

*Tetrahymena geleii* (Furgason, 1940) growing in a wild culture with heavy bacterial growth were concentrated by centrifugation and 1 ml. was introduced into flask No. 1. The migration fluid was 0.5 per cent yeast extract. One milliliter was taken over at each transfer of ciliates from one flask to the next in line. Ten tubes were collected. Migration was excellent and consumed 30 minutes.

The results are tabulated in Table 1.

TABLE 1

*Tetrahymena geleii*

(Whole-Tube Turbidity Test)

| Tube No. | Ciliates Present | Sterile at 48 Hours | Sterile at 72 Hours | Tube No. | Ciliates Present | Sterile at 48 Hours | Sterile at 72 Hours |
|---|---|---|---|---|---|---|---|
| 1..... | o | + | + | 6..... | + | + | + |
| 2..... | o | + | + | 7..... | ++ | + | − |
| 3..... | o | + | + | 8..... | +++ | − | − |
| 4..... | o | + | + | 9..... | +++ | − | − |
| 5..... | o | + | + | 10 ..... | +++ | − | − |

Six organisms were isolated from tube No. 6, 14 from tube No. 7, and 5 from tube No. 8. These were set up in shell vials containing 0.5 per cent sterile yeast extract.

Results are tabulated in Table 2. All plates (Table 2) were prepared in duplicate series, one set incubated at room temperature, and the other at 37° C.

TABLE 2

*Tetrahymena geleii*

| Vial Number | From Tube Number | No Turbidity at 96 Hours | Plates Sterile at 96 Hours | Plates Sterile at 240 Hours | Replatings Sterile at 240 Hours | Vial Number | From Tube Number | No Turbidity at 96 Hours | Plates Sterile at 96 Hours | Plates Sterile at 240 Hours | Replatings Sterile at 240 Hours |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1..... | 6 | + | − | − | − | 15..... | 7 | + | + | + | + |
| 2..... | 6 | + | + | + | + | 16..... | 7 | + | − | − | − |
| 3..... | 6 | + | + | + | + | 17..... | 7 | − | − | − | − |
| 4..... | 6 | − | − | − | − | 18..... | 7 | + | − | − | − |
| 5..... | 6 | + | + | + | + | 19..... | 7 | − | − | − | − |
| 6..... | 6 | − | − | − | − | 20..... | 7 | + | − | − | − |
| 7..... | 7 | + | − | − | − | 21..... | 8 | + | + | + | + |
| 8..... | 7 | + | + | + | + | 22..... | 8 | − | − | − | − |
| 9..... | 7 | − | − | − | − | 23..... | 8 | + | − | − | − |
| 10..... | 7 | + | + | + | + | 24..... | 8 | − | − | − | − |
| 11..... | 7 | + | − | − | − | 25..... | 8 | − | − | − | − |
| 12..... | 7 | + | − | − | − | | | | | | |
| 13..... | 7 | + | − | − | − | % sterile* | ..... | 68 | 32 | 32 | 32 |
| 14..... | 7 | + | + | + | + | | | | | | |

* According to test.

This experiment shows how unreliable turbidity tests (Table 1) for sterility can be. Judging by turbidity, 68 per cent of the selected organisms appeared to be sterile, but agar plates streaked with the broth brought the percentage down to 32 per cent. Transplants from the 8 sterile tubes were heated (after 48 hr. of growth) to 40° C. for 5 minutes to kill the ciliates. These tubes proved to be entirely devoid of living organisms after 3 weeks of incubation at room temperature.

*Glaucoma scintillans* were concentrated from a wild culture, heavily contaminated with bacteria. The organisms were migrated through 0.5 per cent yeast extract in the apparatus. Tubes Nos. 6, 7, 8, 9, and 10 were collected. No ciliates were found in tube No. 6.

TABLE 3

*Glaucoma scintillans*

(Single Isolations from Tube No. 7)

| Tube No. | Ciliates Observed after 79 Hours | Ciliates Present after 127 Hours | Turbidity Tests | Agar Plates | | Tube No. | Ciliates Observed after 79 Hours | Ciliates Present after 127 Hours | Turbidity Tests | Agar Plates | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sterile after 79 Hours | Sterile after 127 Hours | Sterile after 240 Hours | | | | Sterile after 79 Hours | Sterile after 127 Hours | Sterile after 240 Hours |
| 1..... | − | − | + | + | + | 15..... | + | + | + | + | + |
| 2..... | − | + | + | + | + | 16..... | + | + | + | + | + |
| 3..... | − | + | + | + | + | 17..... | + | + | + | + | + |
| 4..... | + | + | + | + | + | 18..... | + | + | − | − | − |
| 5..... | + | + | + | + | + | 19..... | − | + | + | + | + |
| 6..... | − | + | + | + | + | 20..... | − | + | + | + | + |
| 7..... | + | + | + | + | + | 21..... | − | − | + | + | + |
| 8..... | + | + | + | + | + | 22..... | + | + | + | + | + |
| 9..... | + | + | + | + | + | 23..... | − | − | + | + | + |
| 10..... | − | − | + | + | + | 24..... | − | + | + | + | + |
| 11..... | − | − | + | + | + | 25..... | − | + | + | + | + |
| 12..... | − | − | + | + | + | | | | | | |
| 13..... | − | − | + | + | + | % sterile.. | ..... | ..... | 96 | 96 | 96 |
| 14..... | + | + | + | + | + | | | | | | |

Twenty-five ciliates were isolated from tube No. 7 and placed one each in test tubes containing 0.5 per cent yeast extract. No isolations were made from tubes Nos. 8, 9, and 10.

Sixty-five per cent of the selected organisms started thriving[4] cultures, and of these cultures 96 per cent proved to be sterile by turbidity and agar-plate tests. It will be noted here that the turbidity test agrees exactly with the agar-plate tests, whereas in the tests made on *T. geleii* there was marked disagreement. The fact that the division rate of *T. geleii* was much more rapid than *G. scintillans* under the conditions of this experiment probably supplies the answer. In a rapidly growing culture the bacteria may be consumed, so that turbidity tests are misleading. On the other hand, in slow-growing cultures turbidity tests usually are much more reliable. Table 3 shows a considerable

[4] The method of culturing this species, together with its food requirements, will be reported later by Professor Kidder.

lag period for some of the tubes (ciliates were not observed even after 79 hr. but showed up at the end of 127 hr. in numbers sufficient to be observed); and so, if bacteria were present, conditions were very favorable for their rapid growth. The turbidity tests in this case proved to be equally as reliable as the agar-plate test for sterility.

## IV. CONCLUSION

The apparatus and method herein described are not of universal application but have been found adequate for rapidly swimming forms which will rise in a vessel. With further study of various types of protozoa, pH of media, and possible application of electric current during migration it is believed this method will prove useful for a large number of similar species.

As already pointed out, the advantages of such a method are relative simplicity, lack of chance contamination during migration, short time of operation, and number of sterilized organisms recovered.

## V. SUMMARY

1. An apparatus for sterilizing certain protozoa is described.

2. The apparatus takes advantage of both the migration of the protozoa through a sterile fluid and a high dilution factor.

3. Experimental data on four species of ciliates sterilized by this method are given.

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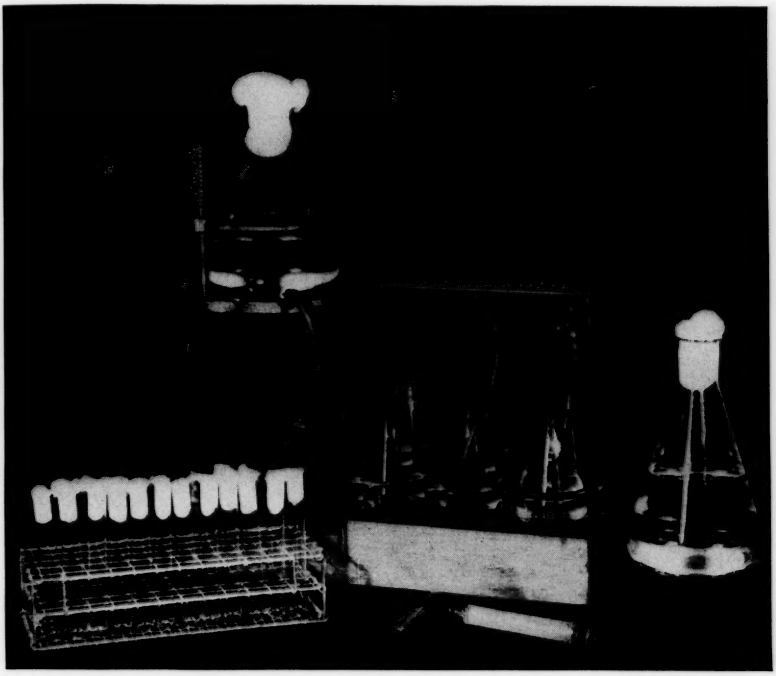
PLATE I

Photograph of assembled migration-dilution apparatus

PLATE I



UNIV. OF MICH.

# THE CRUSTACEAN SINUS GLAND AND CHROMATOPHORE ACTIVATION[1]

(Ten figures)

FRANK A. BROWN, JR.
Department of Zoölogy, Northwestern University, and Marine Biological Laboratory, Woods Hole

IT IS astonishing that so much work has been done upon the effects of eyestalk extract upon chromatophore response and yet the point of origin of the active substance in the stalk has not been determined conclusively. Although a recently published textbook of general physiology states the optic nerve to be the source of the substance, the blood gland (sinus gland) of the stalk has been most generally assumed to be the source since Hanström's (1935) experiments in this regard. In these experiments Hanström divided the eyestalks of various crustaceans transversely and found that the portion yielding chromatophorotropic activity invariably contained, among other things, the sinus gland. This evidence, although moderately convincing, was not conclusive. A nerve tissue, for instance, closely associated with the gland, could well have been the actual source.

Experiments have been performed which demonstrate very clearly the source of chromatophorotropic material in the eyestalk. A minute fragment of glandular tissue, about a hundredth part of the volume of the whole stalk which shows approximately 80 per cent of the chromatophorotropic activity of the whole stalk, has been isolated. Injection of an extract of this minute gland and implantation of the gland into animals from which the gland (eyestalk) had previously been removed give conclusive evidence of its activity.

The gland, from its position in the eyestalk and from the brief histological work that was done upon it (Brown and Cunningham, 1939), appears to be identical with Hanström's (1937) *Sinusdrüse* (blood gland) and is the source of the viability and molt-controlling activity described by Brown (1938) and confirmed by Brown and Cunningham (1939). Hanström's description of the gland in *Cambarus* was so inadequately done that definite identification has been difficult. It seems quite safe, however, to assume their identity and to speak of the gland in the following description as the sinus gland.

## THE SINUS GLANDS OF SEVEN CRUSTACEANS

The sinus glands of each of the crustaceans so far examined (*Callinectes*, *Carcinus*, *Libinia*, *Pagurus*, *Uca*, *Crago*, and *Palaemonetes*) have been easily isolated from the remaining stalk tissue. The gland is quite conspicuous through the transparent exoskeleton of *Crago* and *Palaemonetes*, especially when the normal animal is placed in a strong beam of light and against a black background. In these latter forms it appears in the position indicated in Figure 1, *B* and *F*, as a relatively opaque mass of bluish-white

[1] This investigation was supported in part by a research grant from the Graduate School of Northwestern University.

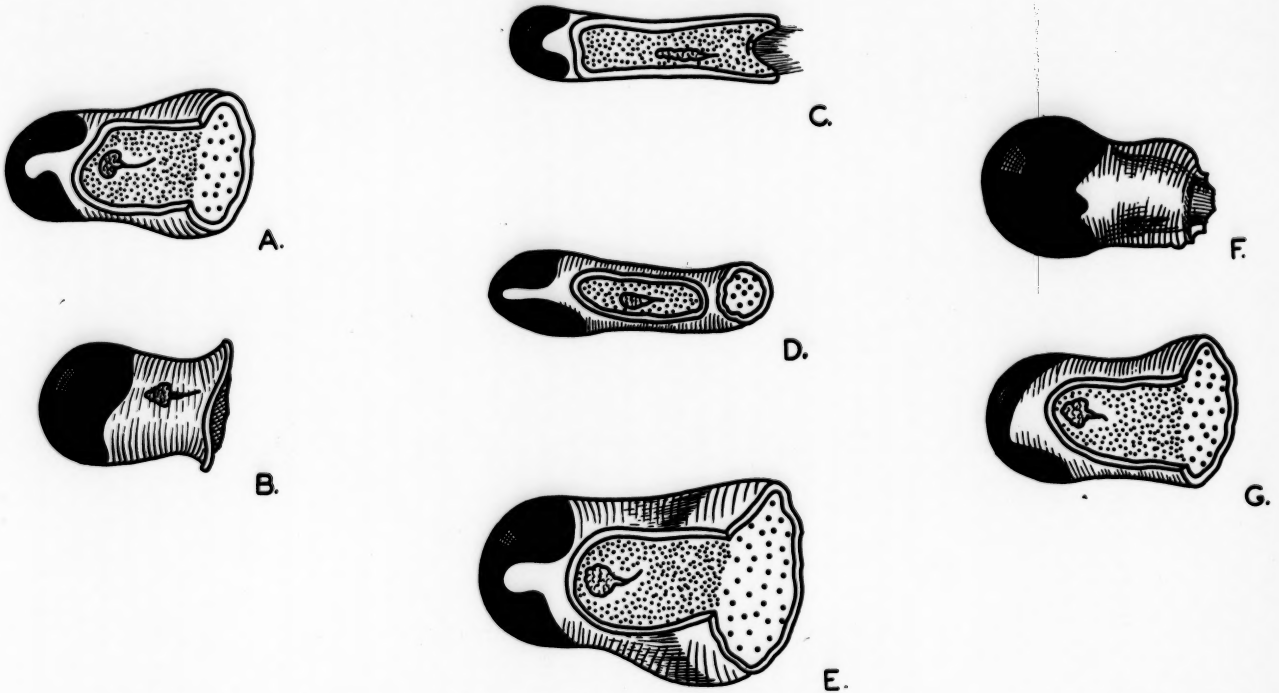



FIG. 1.—The left eyestalks of 7 crustaceans showing more or less of the exoskeleton and hypodermis dissected away to reveal the tear-drop-shaped sinus gland. *A, Carcinus; B, Crago; C, Pagurus; D, Uca; E, Callinectes; F, Palaemonetes; G, Libinia.*

tissue. It is slightly more easily observed in *Palaemonetes* than in *Crago*. In the remaining crustaceans mentioned above, it is not observed until the rather opaque dorsal exoskeleton, together with the pigmented hypodermis, has been removed from the stalk. The gland then stands out very conspicuously as a whitish or bluish-white tissue lying upon the comparatively transparent nervous tissue. It is a simple matter to remove the gland in one piece with watchmaker's forceps under the lower magnifications of a dissecting microscope. Figure 1, *A*, *B*, *C*, *D*, *E*, *F*, and *G*, shows roughly the relative sizes, shapes, and the positions of the glands as they appear in the different crustaceans which have been investigated in this regard. No special attempt was made to draw the different stalks to the same scale. All are left stalks.

In most of the injection experiments to be described the glands were dissected out of freshly excised eyestalks, placed in clean depression slides, allowed to dry, and then thoroughly ground with a glass rod. The ground mass was then extracted with the desired quantity of sea water and injected. In implantation experiments the glands were carefully maintained intact in sea water and were, within 2 or 3 minutes after removal, implanted as wholes into the abdominal region of hosts without sinus glands. By means of a fine glass capillary attached to an aspirator it has also been possible to remove the sinus gland, without apparent further injury to the animal. The results of these latter experiments will form the basis of a future report.

The sinus gland is removed with such ease that there appears to be little need for future investigators to resort to the coarser technique of triturating whole eyestalks when they are dealing with principles of this gland from such crustaceans as are mentioned herein.

### THE SIZE OF THE CRAYFISH SINUS GLAND

To make this determination *Cambarus virilis* were selected, their carapace length measured, and their volume determined by water displacement. An eyestalk was next removed and put into a physiological salt solution. The eyestalk tissue was freed from the inclosing exoskeleton, and the sinus gland dissected out.

TABLE 1

| Expt. No. | Carapace Length (Mm.) | Total-Body Volume ($Mm.^3$) | Eyestalk-Tissue Volume ($Mm.^3$) | Sinus-Gland Volume ($Mm.^3$) | Sinus Gland (Percentage of Eyestalk-Tissue Volume) | 2 Sinus Glands (Percentage of Total-Body Volume) |
|---|---|---|---|---|---|---|
| 1..... | 36 | 13,000 | 2.605 | 0.0250 | 0.95 | 0.00039 |
| 2..... | 36 | 11,500 | 2.932 | 0.0556 | 1.89 | 0.00096 |
| 3..... | 31.2 | 7,500 | 2.000 | 0.0306 | 1.53 | 0.00082 |
| 4..... | 25.5 | 4,800 | 1.446 | 0.0193 | 1.33 | 0.00080 |

The volumes of the sinus gland and remaining stalk tissue were then measured as follows. Each of the pieces of tissue was crushed between two glass slides, which were held apart by fragments of cover slip of known thickness. The outline was traced upon graph paper by means of a camera lucida, and the area calculated. Knowing the thickness of the preparations, the volumes were readily ascertained and expressed in cubic millimeters, percentage of total eyestalk tissue volume, or percentage of total-body volume. The results are given in Table 1.

SINUS GLAND EXTRACT VERSUS WHOLE-EYESTALK EXTRACT

After a few initial experiments had indicated that extracts of the isolated sinus gland had a tremendous chromatophorotropic effect upon *Uca* black chromatophores and *Palaemonetes* red ones, a series of experiments was designed to determine to what extent the chromatophorotropic influence of eyestalk extracts could be attributed to activity of the sinus gland. Sea-water extracts of sinus glands of different crustaceans were made, and each was injected into eyestalkless *Uca* with concentrated black pigment (having a black chromatophore index of one) and eyestalkless *Palaemonetes* with broadly dis-

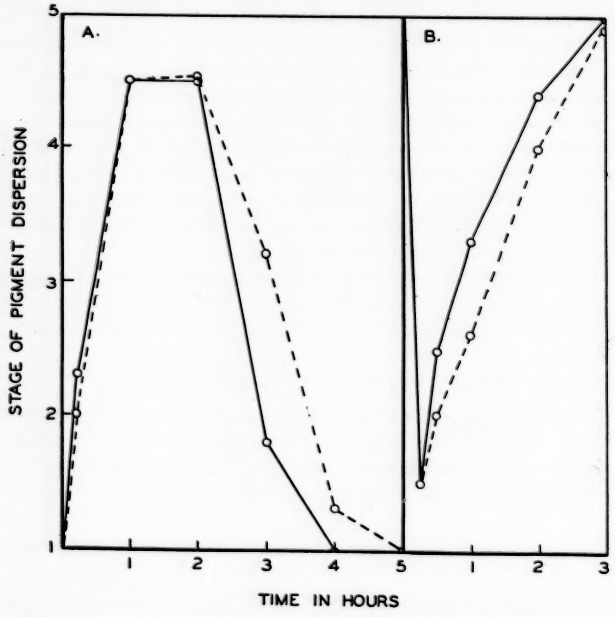



FIG. 2.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Uca* sinus-gland extract (solid line) and *Uca* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

persed red pigment (having a red chromatophore index of five). In each case the same extract was injected into 10 *Uca* and 10 *Palaemonetes*. The dose given was always 0.05 cc. into *Uca* (at base of fourth walking leg) and 0.025 cc. into *Palaemonetes* (dorsal abdominal musculature). The concentration in the instance of *Uca* sinus gland was 1 sinus gland to 1 cc. of sea water, whereas in the other instances the concentration was 4 sinus glands to 1 cc. of solution.

The effects of injection of the extracts were measured quantitatively for the two types of chromatophores, using the change in the average chromatophore index with time as the criterion. For the red chromatophores of *Palaemonetes* their state was judged at 15, 30, 60, 120, and 180 minutes after extract injection. With *Uca* black chromatophores their state was judged at 15, 60, 120, 180, 240, and 300 minutes after injection.

The chromatophore-index method was essentially that employed by Hogben and his school (see Hewer, 1923; Hogben and Slome, 1931). A fully dispersed pigment mass was designated as 5, and a fully concentrated mass as 1. Different intermediate degrees of dispersion were called 2, 3, and 4, depending upon the degree. As an indication of the effect of an extract upon *Uca* black chromatophores, the sum of the average indices for each time interval was obtained, and the difference between it and 6 (no effect) was found. For the effect upon *Palaemonetes* red pigment the difference between 25 (no effect) and the sum of the average indices for each time interval was found. Such an index of the

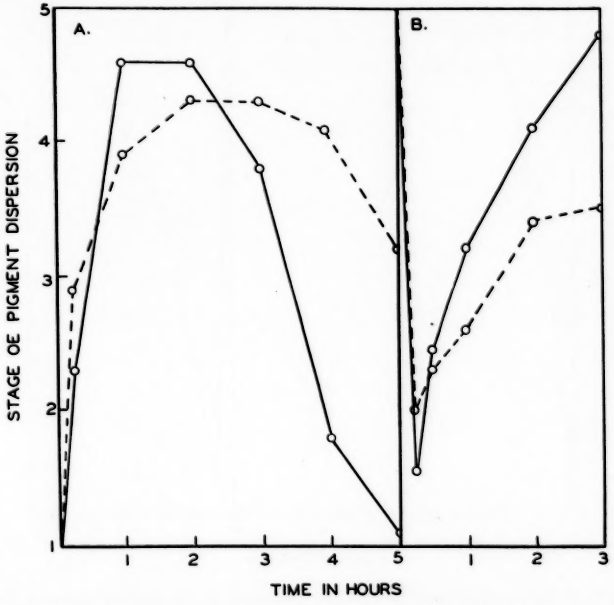



FIG. 3.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Carcinus* sinus-gland extract (solid line) and *Carcinus* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

total effect of an extract (the index value in the following descriptions) was believed reasonable, since it took into consideration both the magnitude of the chromatophore change and the duration of the effect.

The effects that were obtained with sinus-gland extracts were then compared with effects of whole-eyestalk extracts in which the extracts were prepared in the same way as for sinus glands, except that whole eyestalks were substituted for sinus glands. In so far as was possible, animals of similar sizes and weights were used with both sinus-gland and eyestalk experiments. Owing to lack of time, usually only 5 animals were injected. The experiments with each source of sinus gland and eyestalk will be discussed in turn.

*Uca*.—Four sinus glands were removed from 2 *Uca* weighing a total of 3.40 gm.,

and extracted in 4 cc. of sea water. This extract was injected into 10 *Uca* (0.05 cc. to each), having an average weight of 1.86 gm. Four eyestalks were removed from 2 *Uca*, weighing a total of 3.35 gm., and an extract of them injected into 10 *Uca* which had an average weight of 1.69 gm.

The same extracts were also injected into 2 lots of large female *Palaemonetes*, 7 animals to each lot. The *Palaemonetes* were roughly all the same size, though their weights were not determined.

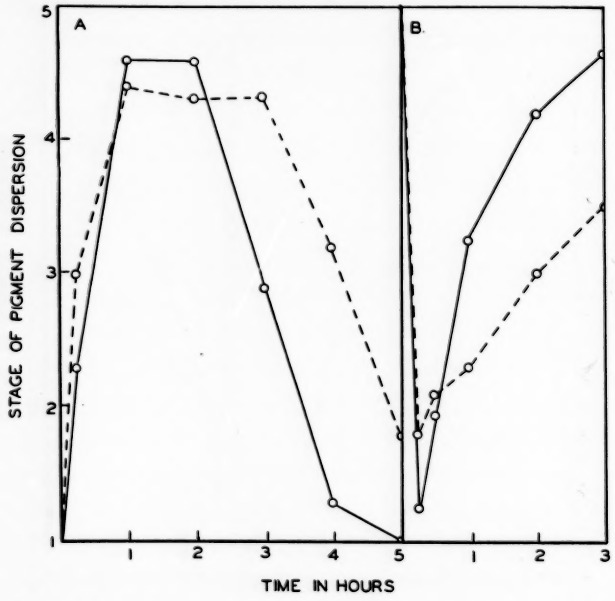



FIG. 4.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Pagurus* sinus-gland extract (solid line) and *Pagurus* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

The results of these experiments are graphically shown in Figure 2. It is seen that the index of effect of sinus gland upon *Uca* was 9.1 and of whole eyestalk was 10.5. Thus the effect of the sinus gland by itself was 86.7 per cent of that of the whole stalk. Similarly, on *Palaemonetes* the sinus-gland–effect value was 7.7 as against 9.2 for the whole stalk. Here the sinus gland showed 83.7 per cent of the effect of the whole stalk.

*Carcinus*.—The average weight of the animals from which the sinus glands were removed was 56.4 gm. The average weight of the donors of the whole stalks was 56.0 gm. Each was made into an extract of 4 glands or stalks to 1 cc. of sea water. The effects of these extracts when injected into eyestalkless *Uca* and *Palaemonetes* are seen in Figure 3. On *Uca* the index value (I.V.) for sinus gland was 12.2 and for eyestalk 16.7. Here the sinus gland showed 73 per cent activity of the whole stalk. For *Palaemonetes*

red pigment the index value of sinus-gland activity was 8.9 and for eyestalk 11.2. Here the sinus gland showed 79.5 per cent of the activity of the whole stalk.

*Pagurus*.—Four sinus glands from animals with an average weight of 9.6 gm. and 4 eyestalks from animals of average weight of 10.1 gm. were extracted in concentration of 4 glands or stalks to 1 cc. sea water. The I.V. of the effect of the sinus gland on *Uca* was 10.6 and of whole eyestalk 15.0. Sinus gland thus showed 70.6 per cent activity of whole stalk. With *Palaemonetes*, I.V. of sinus gland was 9.7 and of eyestalk 12.3, with

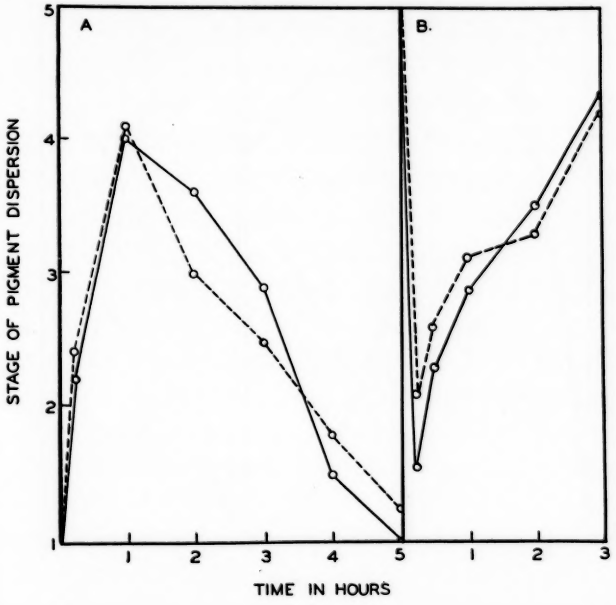



FIG. 5.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Palaemonetes* sinus-gland extract (solid line) and *Palaemonetes* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

sinus gland showing 79 per cent of total stalk activity. The results are plotted in Figure 4.

*Palaemonetes*.—The *Palaemonetes* from which the stalks and glands were removed weighed about 1 gm. apiece. The extracts of whole eyestalks and sinus glands alone were made in the usual concentrations. The effects were I.V. of eyestalk on *Uca*, 9.0; sinus gland on *Uca*, 9.2. Here the sinus gland showed even more effect than the whole stalk. This result is undoubtedly within the error of the method. On *Palaemonetes* red pigment I.V. of eyestalk was 9.7 and of sinus gland 10.4. Here again the sinus gland was more effective than the whole stalks. The results are shown in Figure 5.

*Libinia*.—Four sinus glands were removed from *Libinia* of average weight of 73.3 gm., and 4 eyestalks from *Libinia* of average weight of 83.5 gm. Extracts were made using

4 glands or stalks to 1 cc. of sea water. On *Uca* black pigment the I.V. of effect of eyestalk was 18.4 and of sinus gland was 10.7. Sinus gland thus showed 58.2 per cent of activity of whole stalks. On *Palaemonetes* red pigment the I.V. for eyestalk was 13.6 and of sinus gland 11.3. Sinus gland thus showed 83.2 per cent of total activity of the whole stalk. These results are seen graphically in Figure 6.

*Callinectes*.—Four sinus glands were removed from *Callinectes* of average weight of 87.5 gm. and 4 eyestalks from animals of average weight of 124 gm. These were ex-

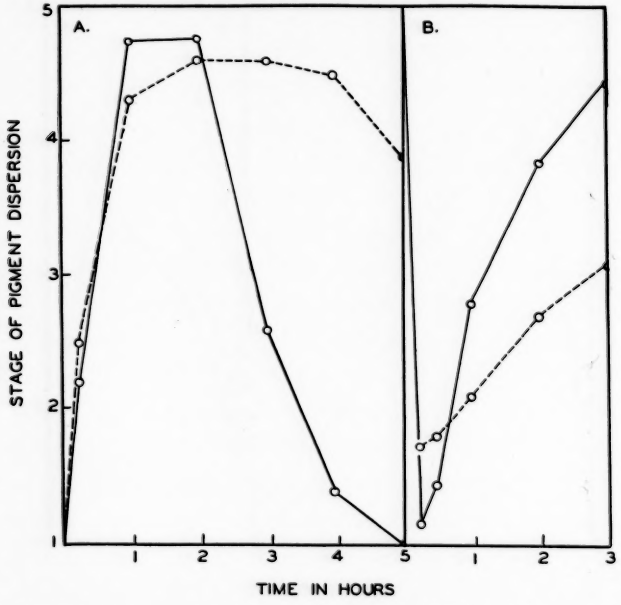



FIG. 6.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Libinia* sinus-gland extract (solid line) and *Libinia* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

tracted in sea water in concentration of 4 stalks or glands to 1 cc. The I.V. of effects on *Uca* black were eyestalk, 16.4; sinus gland, 12.3. Thus sinus glands showed 75 per cent of total stalk activity. On *Palaemonetes* red pigment the I.V. of effects were eyestalk, 14.0; sinus gland, 13.2. Here sinus gland showed 94.2 per cent of total stalk activity. Figure 7 shows the results of this experiment.

*Crago*.—Four sinus glands and 4 eyestalks were removed from *Crago* weighing between 0.8 and 1.0 gm. each. These were extracted in sea water in the concentration of 2 glands[2] or stalks to 0.5 cc. The I.V. of effect on *Uca* black pigment was eyestalk, 13.7; sinus gland, 10.7. Sinus gland showed 78.1 per cent of total stalk activity. Strangely

[2] Because of the difficulty of removal of this organ in *Crago*, there is some question of the exact concentration of the extract. At any rate, it lies between the concentration indicated and a value 50 per cent greater.

enough, these same two extracts when injected into *Palaemonetes* showed I.V. effect of eyestalk as only 0.2 while the sinus gland showed 4.0. These results are seen in Figure 8.

*General.*—Taking all the results together but excluding the response of *Crago* tissues upon *Palaemonetes*, the value of which for some reason falls completely out of line with the remainder, the average percentage of total chromatophorotropic activity of the eyestalk to be found in the minute sinus gland by itself is 82.5 per cent. This leaves a

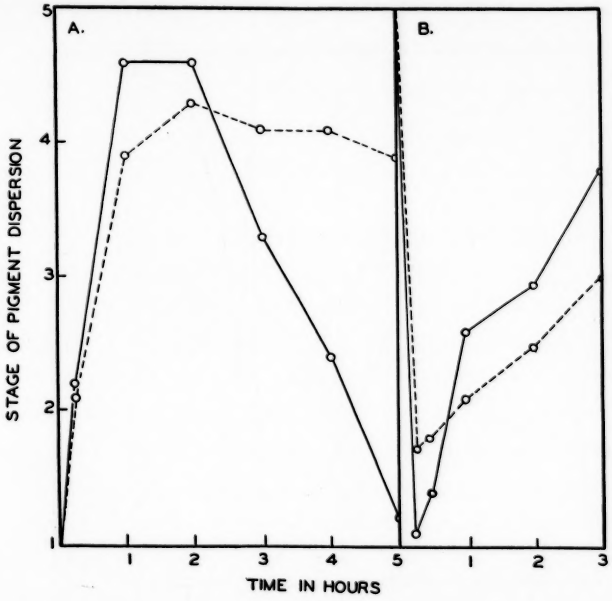



FIG. 7.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Callinectes* sinus-gland extract (solid line) and *Callinectes* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

residue of activity of the stalk of 17.5 per cent. There seems to be no reason to suspect that the difference of 17.5 per cent need be accounted for by anything other than the normal diffusion of materials from the gland to other eyestalk tissues during the course of gland activity. It is also unlikely that the gland is removed without any loss of some of its constituent cells or cell contents.

### SINUS-GLAND EXTRACTS VERSUS EXTRACTS OF STALK REMAINDER

General confirmation of the preceding results was obtained by comparing the effects of sinus-gland extracts of *Carcinus* with effects of extracts of the remaining stalk tissue extracted in the same quantity of sea water. The only test employed in this experiment was the red pigment of eyestalkless *Palaemonetes*.

The extracts were made up as absolute ethyl alcohol extracts of dried tissue according to the method of Abramowitz (1936). The ultimate concentrations used were 1 sinus-glandless stalk or 1 sinus gland to 0.1 cc., and these same extracts diluted ten times. The dose injected into the test animals (large eyestalkless female *Palaemoneest*) was always 0.02 cc., and 5 animals were injected to determine the I.V. of the particular extract.

The results are seen in Figure 9. It is readily calculated that at the stronger concentration the I.V. of effect of the sinus gland is 12.4 and the glandless stalk 7.3. Thus, of

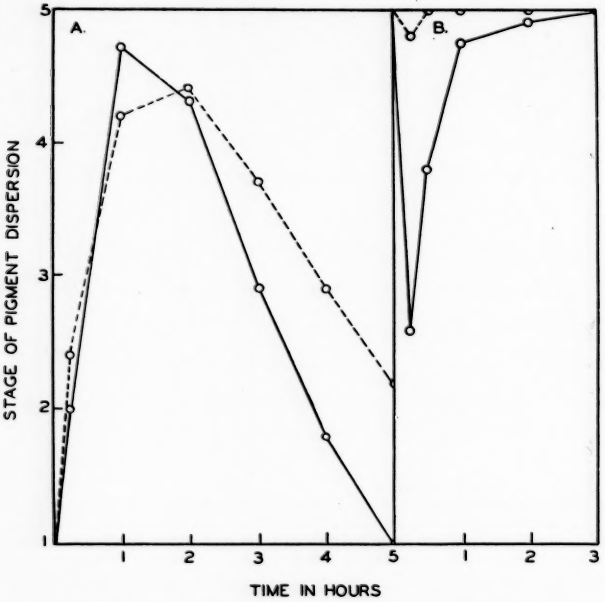



FIG. 8.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (*A*) and the red pigment of *Palaemonetes* (*B*), following injection of *Crago* sinus-gland extract (solid line) and *Crago* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

the whole stalk, the sinus gland is showing 63 per cent of the total effect, and the remainder of the stalk tissue 37 per cent. At the greater dilution these proportions are respectively 97 per cent and 3 per cent. The average of these two values indicates that 80 per cent of the total stalk chromatophorotropic effect was in the sinus gland proper and that the remainder of the stalk tissue showed 20 per cent. This is in remarkable agreement with the average results of the earlier experiments performed in a slightly different manner that gave the values 82.5 per cent and 17.5 per cent and more particularly in agreement with the results of *Carcinus* extracts on *Palaemonetes* which gave the values 79.5 per cent and 20.5 per cent. The apparent exact repeatability of the

*Carcinus* results can scarcely be attributed to more than chance coincidence, considering the type of experiment.

### CHROMATOPHOROTROPIC ACTIVITY OF SINUS-GLAND IMPLANTS

Sixty large female *Palaemonetes* were deprived of their eyestalks. The red pigment took up its usual state of complete dispersion. At the end of 24 hours the animals were divided into two lots of 30 each. Into the ventral region of the last abdominal segment of each animal of lot *A* was implanted a whole *Carcinus* sinus gland by means of a fine

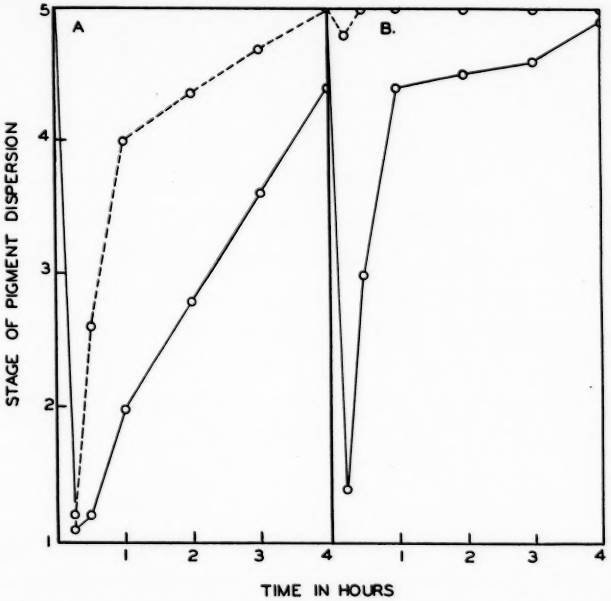



FIG. 9.—Comparative effects of injection of extracts of sinus gland (solid line), and of sinus-glandless eyestalk (broken line) upon the red pigment of *Palaemonetes* as it appears when using 1 glandless stalk or 1 gland to 0.1 cc. of sea water (*A*) and 1 glandless stalk or 1 gland to 1 cc. sea water (*B*).

glass pipette which was inserted at the base of a uropod. Each animal of lot *B* received a similar puncture and an equivalent dosage of sea water, but no gland. Each of the animals of lot *A* showed the sinus-gland implant clearly visible through the transparent exoskeleton and became lighter and bluish almost immediately as if they had received an injection of extract of the gland. Lot *B*, the controls, showed no chromatophoric response. At the end of 1, 3, 5, and 10 days, the average I.V. of the state of red-pigment dispersion of each lot was determined. These are plotted in Figure 10. The results indicate that the effect of the implant is to concentrate the red pigment and that the effect is maximum during the first day or two and then gradually wears off during 6 or 7 days. Interestingly enough, this is just about the extent of lengthening of the life of

sinus-glandless animals by sinus-gland implants (Brown, 1938; Brown and Cunningham, 1939). In individual animals there was an interesting correlation observed between the time of loss of chromatophoric influence and the time the gland ceased to maintain its clear bluish-white appearance. Those glands which came to lie directly alongside the ventral nerve cord were those that maintained their activity longest.

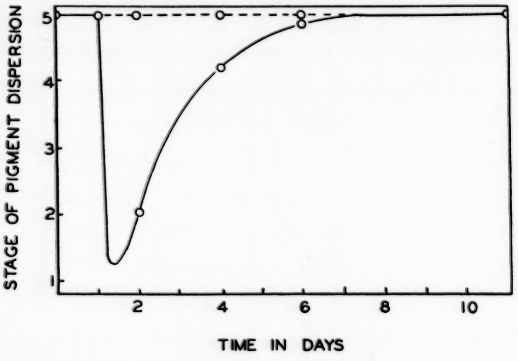



FIG. 10.—The average stage of dispersion of red pigment in two lots of 30 eyestalkless *Palaemonetes*, into one lot of which *Carcinus* sinus glands were implanted into the abdomen after 1 day (solid line), the other lot receiving a comparable puncture but no implant (broken line.)

The white chromatophores showed no significant difference in the two lots, the average white chromatophore I.V. of the two lots at 1, 3, and 5 days after implantation was, with implant, 1.9; and without it, 2.0; with no consistent change during those 5 days.

## CONCLUSIONS

A definite glandular body, the sinus gland, has been located in the fresh eyestalk of seven crustaceans, *Callinectes*, *Libinia*, *Carcinus*, *Pagurus*, *Crago*, *Palaemonetes*, and *Uca*. From all of these forms the gland is easily removed by dissection, and in two of them, *Crago* and *Palaemonetes*, it can be clearly seen in the normal living animal under suitable lighting. The gland appears to be the sole tissue of the eyestalk which secretes a significant quantity of chromatophorotropic material influencing the red pigment cells of *Palaemonetes* and the black ones of *Uca*. Although the gland constitutes only about a hundredth part of the total-stalk tissue, its effects have been shown to be 80 per cent or more of that of the whole stalk, and there is no justification for belief that the remaining 20 per cent of the activity originates from any other tissue but rather results from normal and artificially induced escape of material from the gland proper. These results have been obtained from three different types of experiments: (1) quantitative comparison of effects of sinus-gland extracts with those of the whole-eyestalk extract; (2) quantitative comparison of effects of sinus gland by itself with effects of eyestalk tissue from which only the sinus gland had been removed; and, finally, (3) implantation of sinus glands into the ventral abdominal region of animals having their sinus glands removed.

All the crustaceans examined have the same type of gland, judging from its gross

appearance and from its location. All the sinus glands have principles effective on *Uca* black pigment and on *Palaemonetes* red pigment. Furthermore, for each species the relative effects of its sinus gland as compared with its whole eyestalk upon the *Uca* black or *Palaemonetes* red pigment have been generally the same. Thus, at least in the majority of crustaceans, the whole eyestalk and sinus gland have qualitatively the same effect according to the criterion used. This further eliminates the possibility that another eyestalk gland is affecting either of these chromatophore types, unless it functions in the same manner as the sinus gland.

Summarizing briefly, the evidence indicates that the source of the chromatophorotropic substance of the crustacean eyestalk is the sinus gland. As far as the *Uca* black chromatophores and the *Palaemonetes* red ones are concerned, this gland will account wholly for the activity of the eyestalk extracts.

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# THE EFFECT OF CONCENTRATION OF COLPODA DUODENARIA ON THE TIME REQUIRED FOR ENCYSTMENT IN FOOD-FREE MEDIUM

(Four figures)

A. G. R. STRICKLAND
Stanford University

THE resting cysts of *Colpoda duodenaria* offer promising material for experimental studies, both qualitative and quantitative, on protoplasm in a relatively dedifferentiated state. In order to be certain that the material prepared at different times can be considered as physiologically the same, it is necessary to know which environmental factors must be controlled during the growth of the cultures and the preparation of the cysts.

It has been shown (Taylor and Strickland, 1938, 1939) that

1. The formation of resting cysts in *C. duodenaria* follows the reduction in amount of the available food supply in the medium.

2. Those organisms which do not form resting cysts in the absence of food will form "rhythmic" cysts, i.e., the *Colpoda* will encyst but will spontaneously reorganize and excyst, and this process will be repeated until, owing to depletion of the body substance, death ensues.

3. The percentage of resting cysts formed varies directly with the logarithm of the concentration of the protozoa.

4. The percentage of resting cysts formed at constant concentration of organisms decreases as the volume of medium decreases between $\frac{1}{4}$ and 1/40,000 cc. It seems that the protozoa condition the medium, promoting thereby the change from cyst to resting cyst. It is of importance to know at what stage of the process of encystment this interaction among the *Colpoda* begins to affect them. This paper shows the effect of the concentration of the organisms on the duration of the precyst period of *Colpoda* in various volumes of food-free medium.

## MATERIAL AND METHODS

The protozoa, food, culture medium, and much of the technique were as described elsewhere (Taylor and Strickland, 1938, 1939). About one million resting cysts in a dish were washed to remove unattached debris and then induced to excyst by the addition of yeast extract. As soon as the first free-swimming *Colpoda* were seen, the yeast extract was poured off, and balanced medium added. Forty-five minutes later, when excystment was almost complete, the culture was decanted into a clean dish and fed with that amount of a suspension of bacteria determined by experience, which will allow the organisms to grow large enough to form quadrigenic division cysts.

The resulting culture of approximately four million *Colpoda* was washed twice in the centrifuge to remove cyst membranes and most of the bacteria. About fifty thousand of these washed protozoa in $\frac{1}{4}$ cc. of medium were put into each of a number of Colum-

bia dishes. They formed resting cysts, which adhered so firmly to the clean glass surface that they could be thoroughly washed as often as required with sterile balanced medium. The cysts in these Columbia dishes provided uniform material for later experiments.

A rigid time schedule was followed. For each series of tests the cysts in one of the Columbia dishes were thoroughly washed before yeast extract was added. After a given period the yeast extract and the first few excysted protozoa were poured off and replaced by sterile balanced medium. After a fixed interval the excysted *Colpoda* were washed in 100 cc. of sterile balanced medium in the centrifuge to remove the cyst membranes. The washing was repeated, and the *Colpoda* thus obtained were sufficiently clean to be regarded as food free.

The end of the above washing procedure was arbitrarily chosen as time "zero," from which to reckon the duration of the precyst period. The end of the precyst period was arbitrarily determined as the moment when the encysting organism, having withdrawn the cilia through the newly secreted ectocyst, began to rotate within the membrane. This, of course, is not the end of the encystment process.

Micropipettes were calibrated to 1/40, 1/200, 1/1,000, and 1/5,000 cc. To obtain drops of 1/25,000 cc., it was found possible to use a 1/5,000-cc., pipette and to distribute its contents in five apparently equal drops with sufficient speed and accuracy. For drops of 1/40 and 1/200 cc. Petri dishes with closely fitting covers were used as observation chambers. For smaller drops, cover slips reversed onto hollow-ground slides were used. For all cases a saturated atmosphere was maintained by additional drops of distilled water.

Five thousand organisms in a drop proved to be the greatest number whose encystment could be followed with sufficient quantitative accuracy. The required number of *Colpoda* was injected into the drops of 1/40 and 1/200 cc. by means of injection pipettes of extremely small caliber. In order not to increase appreciably the volume of the drop when 5,000 organisms were injected into it, the washed *Colpoda* were momentarily concentrated to many millions per cubic centimeter before they were picked up by the injection pipette.

For drops of 1/1,000 cc. and smaller, the food-free *Colpoda* were diluted to approximately the concentration desired, and the requisite number of the protozoa were then picked up with the calibrated pipettes.

These small drops were placed on cover slips, which were immediately reversed onto hollow-ground slides, so that decrease in volume by evaporation was not appreciable. During the distribution of the smaller drops the relative humidity in the constant-temperature room (20° C.) was around 90 per cent.

## EXPERIMENTAL

With the material and by the methods described in the previous section, the encystment was observed of concentrations of *Colpoda* between 1,000 per cubic centimeter (25 per drop) and 200,000 per cubic centimeter (5,000 per drop) in drops of 1/40 cc. Cysts were always noted first in the drops with the highest concentration, but the drops with lower concentrations followed closely with one or more cysts. This is of interest because it shows that at all concentrations encystment begins approximately at the same time.

From five to seven counts of the cysts in each drop of the series were made during encystment. The mean of the times at the beginning and end of the counting period was noted as the time of the observation; fractions of a minute were not noted. An example of such a series is given in Table 1 and is plotted on linear paper in Figure 1;

TABLE 1

SERIES D.78: ENCYSTMENT OF DIFFERENT CONCENTRATIONS IN DROPS OF 1/40 CC
(Time in Minutes from Zero)

| No. | Time | Cysts | Percentage | Time | Cysts | Percentage | Time | Cysts | Percentage |
|---|---|---|---|---|---|---|---|---|---|
| 1..... | 100 | 100 | 2.0 | 122 | 573 | 11.4 | 140 | 2,000 | 40.0 |
| 2..... | 103 | 20 | 2.0 | 128 | 120 | 12.0 | 144 | 252 | 25.2 |
| 3..... | 105 | 2 | 0.7 | 131 | 19 | 7.0 | 150 | 32 | 11.9 |
| 4..... | 136 | 1.5 | 4.8 | 155 | 2.5 | 8.1 | 175 | 4 | 12.9 |
| 1..... | 163 | 3,500 | 70.0 | 180 | 4,400 | 88.0 | 205 | 4,900 | 88.0 |
| 2..... | 165 | 500 | 50.0 | 183 | 750 | 75.0 | 206 | 900 | 90.0 |
| 3..... | 170 | 82 | 30.4 | 190 | 153 | 56.7 | 212 | 198 | 73.4 |
| 4..... | 198 | 7.5 | 24.2 | 218 | 13 | 42.0 | 260 | 19 | 61.3 |

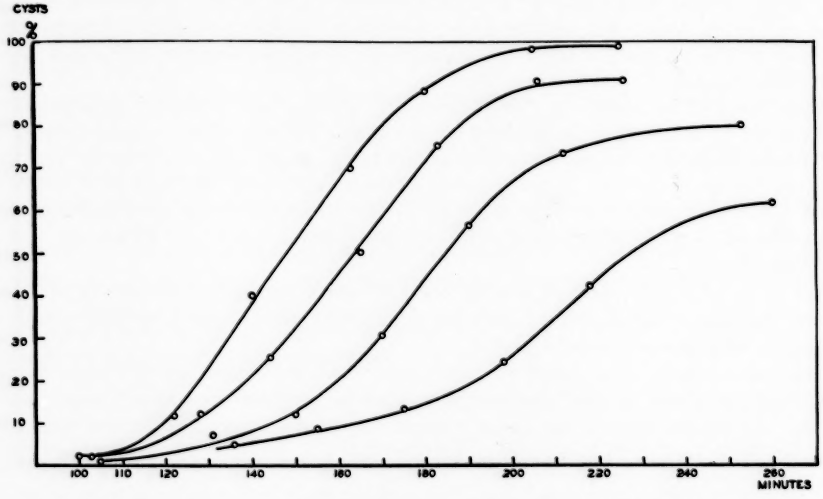


FIG. 1.—Series D. 78. Encystment curves at different concentrations of *Colpoda*. Volume of the drop: 1/40 cc. Ordinates: percentage of cysts at time of observation; abscissae: time of observation in minutes from zero.

the ordinates are the number of cysts per cent of the number of organisms in the drop; the abscissae are the times of observations in minutes from zero.

Encystment evidently proceeds more rapidly as the concentration of organisms increases. In those cases in which the concentration of *Colpoda* is not sufficient to induce

all the protozoa to form resting cysts (Taylor and Strickland, 1939) the curves may never reach the 100 per cent line, because the first animals to encyst may be ready to emerge before all the organisms have encysted. The upper ends of the curves then deviate from the sigmoid form. But, if we take as a criterion of the effect of the concentration

TABLE 2

$t_{50}$ FOR CONCENTRATIONS OF SERIES D.78

| No. | *Colpoda* per Drop | *Colpoda* per Cubic Centimeter | $t_{50}$ |
|---|---|---|---|
| 1..... | 5,000 | 200,000 | 150 |
| 2..... | 1,000 | 40,000 | 164 |
| 3..... | 270 | 10,800 | 185 |
| 4..... | 31 | 1,240 | 230 |

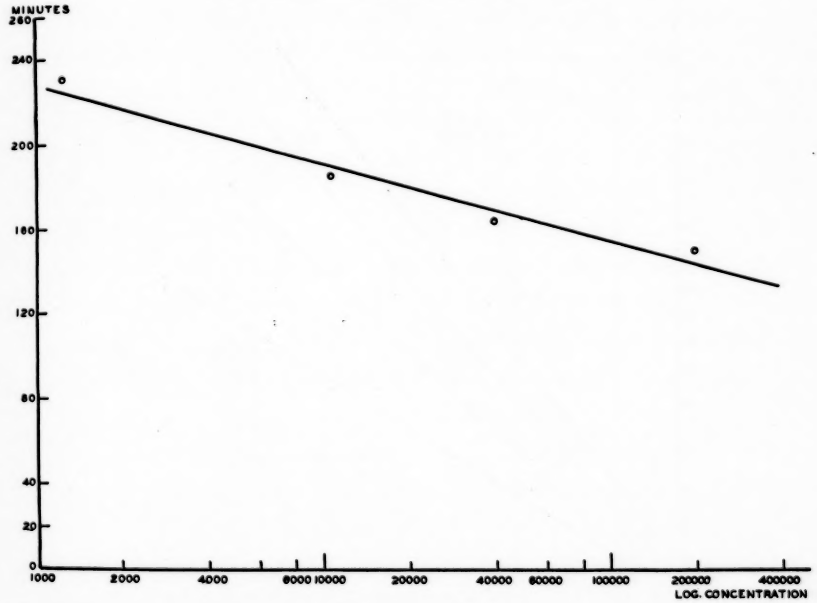


FIG. 2.—Series D. 78. $t_{50}$ at different concentrations of *Colpoda*. Volume of the drop: 1/40 cc. Ordinates: $t_{50}$ in minutes from zero; abscissae: logarithm of the concentration of *Colpoda*.

of protozoa on the rapidity of encystment the time required for the encystment of 50 per cent of them, the curves above that point are not so important.

If these times, $t_{50}$, are taken (Table 2) from Figure 1 and plotted (Fig. 2) against the logarithm of the concentration, it appears that the time required for 50 per cent of the *C. duodenaria* to form cysts in a drop of a given volume is an exponential function of the

TABLE 3

MEAN VALUES OF $t_{50}$ AT DIFFERENT CONCENTRATIONS IN DROPS OF 1/40 CC.

| Group | Cases | Concentration | $t_{50}$ | Standard Deviation | Standard Error |
|---|---|---|---|---|---|
| 1..... | 11 | 1,460 | 201 | 33.3 | 10.0 |
| 2..... | 14 | 9,880 | 174 | 26.4 | 7.1 |
| 3..... | 8 | 45,650 | 156 | 21.6 | 7.6 |
| 4..... | 10 | 198,000 | 151 | 15.0 | 4.7 |

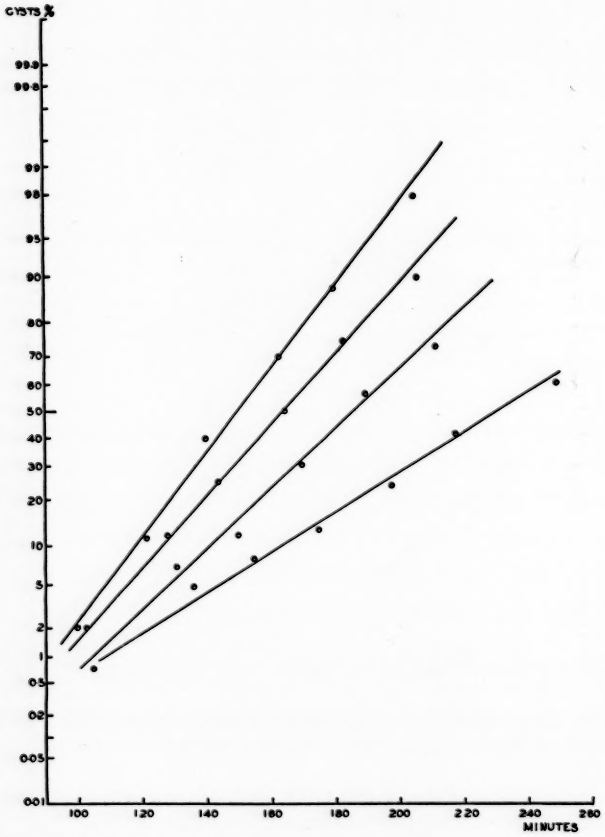


FIG. 3.—Series D. 78. Encystment curves at different concentrations of *Colpoda*. Volume of the drop: 1/40 cc. Ordinates: percentage of cysts (probability scale); abscissae: time of observation (arithmetic scale).

concentration of the protozoa. Thus, for the series shown in Figure 1 the time required for encystment of 50 per cent of the *Colpoda* in 1/40 cc. of food-free medium decreases by about 11 minutes when any concentration is doubled.

At every repetition of the experiment this logarithmic relationship was substantiated, but as the curves for each series did not exactly coincide, probably significant of some as yet imperfectly controlled factors in the technique or environment, eight final series were tested. As nearly as possible the same concentrations were studied in each series, so that the data for the 43 cases could be assembled in four groups.

TABLE 4

ASSEMBLY INTO CONCENTRATION GROUPS OF THE STANDARD DEVIATIONS OF $t_{50}$ FOR ALL VOLUMES OF THE DROP

| Group | Concentration | Standard Deviations | Mean Standard Deviation *M* | Standard Deviation of *M* |
|---|---|---|---|---|
| I..... | 1,000–6,000 | 33.3<br>24.1<br>19.3 | 25.6 | 5.82 |
| II..... | 6,000–<br>50,000 | 26.4<br>26.4<br>19.6<br>26.2<br>21.6 | 24.0 | 2.32 |
| III..... | 50,000–<br>300,000 | 15.0<br>12.7<br>19.9<br>14.4<br>17.2 | 15.8 | 2.49 |
| IV..... | 300,000–<br>2,000,000 | 17.5<br>16.2<br>12.0<br>15.4<br>10.8 | 14.4 | 2.55 |
| V..... | 2,000,000–<br>10,000,000 | 10.9<br>10.5 | 10.7 | 0.2 |
| VI..... | Above<br>10,000,000 | 2.6 | 2.6 | ..... |

Table 3 gives the data for the four groups in 1/40 cc., and Figure 4 shows the straight line through the mean value of $t_{50}$ at the mean concentration for each group.

In some of these cases, especially in those containing few *Colpoda*, it was difficult to determine on linear paper the correct position of the sigmoid curves and so of $t_{50}$. The data were therefore plotted on arithmetic-probability paper (Hazen, 1914; Whipple, 1916), which transforms the curves resulting from random distribution or variation of physiological factors into straight lines (Fig. 3). These lines are more easily determined than the sigmoid curves, though it may be necessary to ignore data above 65 per cent if excystment has begun.

Eight similar series with 55 cases were made in drops of 1/200 cc. Results similar to those for 1/40 cc. were obtained. The range of concentrations is between 1,600 and 1,000,000 *Colpoda* per cubic centimeter (5,000 per drop). The straight line through the mean values of $t_{50}$ at the mean concentration for each of the four groups is shown on semilogarithmic paper in Figure 4. It is nearly, but not quite, the same as for 1/40 cc.

For drops of 1/1,000 cc. there are 145 cases in four groups. In the three smallest sizes of drops each organism represents a considerable concentration per cubic centimeter. In the lower concentrations in these small drops there are not enough organisms to determine adequate encystment curves, so that in each series 5–10 cases containing

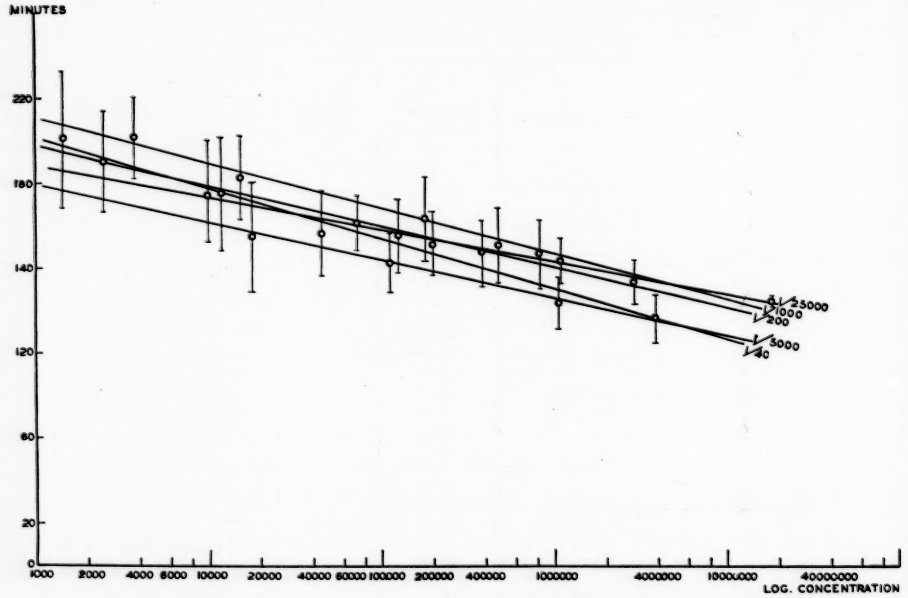


FIG. 4.—Synopsis of mean $t_{50}$ curves for the five different volumes of the drop, with the standard deviations of $t_{50}$ from the mean value of $t_{50}$ for each of the twenty groups. Ordinates: $t_{50}$ in minutes from zero; abscissae: logarithm of the concentration.

the same low concentration of *Colpoda* were observed; these cases were then combined in a subgroup to give adequate data for a reasonably accurate curve. From these subgroups values of $t_{50}$ were obtained, and they, weighted with the number of cases in their subgroups, were used to determine the mean value of $t_{50}$ for the low-concentration group.

Similar experiments were made in drops of 1/5,000 cc. (seven series with 84 cases in four groups), and in drops of 1/25,000 cc. (four series with 100 cases in five groups). The mean curves showing the relationship between $t_{50}$ and the concentration of the protozoa are also shown in Figure 4; they are near, but do not coincide with, those for the other sizes of drop. The standard deviation of the mean of each group was calculated for all five sizes of drop and is represented in Figure 4 by a vertical line extending to equal distances above and below each point.

If the points in Figure 4 are divided into six groups of concentrations and if the mean $M$ of their mean deviations is determined for each group, it will be seen (Table 4) that (1) these standard deviations are of approximately the same order of magnitude within each group, i.e., the standard deviations of $M$ are small; and (2) $M$ increases as the concentration decreases. Since the standard deviations of the points within each group (Fig. 4) all overlap each other and are of about the same magnitude within each group, it must be supposed that under ideal conditions of technique and environment the five curves would coincide. This means that the time required for 50 per cent of the organisms to encyst in food-free medium is independent, within the range here considered, of the volume of the drop containing them.

## DISCUSSION

*Colpoda duodenaria*, regardless of its size, will, in food-free medium, proceed to form a cyst after a certain delay. Part of this delay may be due to the presence of undigested food, which is therefore equivalent to food in the external environment. It is as yet undecided, whether this ciliate stores any such substances as oils or glycogen. This seems improbable, because such reserves should act as inhibitors of encystment to prolong the pre-encystment delay in food-free medium. Preliminary experiments indicate that this delay in a given concentration of *Colpoda* is remarkably constant, regardless of the size and previous feeding of the organism.

Taylor and Strickland (1939) have shown that certain environmental conditions, namely, the concentration of the organisms during encystment and also the volume of the drop of food-free medium in which they are contained, determine the percentage of the cysts that will become resting cysts. It is important to know if these same conditions also influence the precyst delay and the dedifferentiation period of all the organisms, regardless of their subsequent fate. If so, then resting cysts might be physiologically different according to the concentration of the organisms and perhaps the volume of medium in which they were formed.

The selected criterion of the influence of these factors on the precyst period, the time $t_{50}$ between the end of the washing procedure, arbitrarily chosen as zero, and the moment when the encysting protozoön begins to rotate within the ectocyst, can be determined with sufficient accuracy on arithmetic-probability paper. Theoretically, the encystment curve of strictly uniform organisms is a vertical line. The probability scale, representing the normal law of error, tends to eliminate the effect on the sequence of observations of the random distribution of uncontrollable differences in the physiological condition of the *Colpoda* at the beginning of the experiments.

Throughout the process of evolving a satisfactory technique the results obtained, showing the relationship between the concentration of *Colpoda* and the time required for 50 per cent of them to become cysts, were qualitatively the same, though quantitatively somewhat variable. The straight lines on semilogarithmic paper were always straight lines. Some were parallel but shifted by a few minutes on the axis of the ordinates; it seems quite probable that this was due in part to the indeterminate position of zero.

However strictly the time schedule be adhered to, there may be certain unspecifiable factors, temporary promotors or inhibitors of encystment, that might induce a variation in the mean physiological condition of the organism in different experimental series. Such a factor might be the degree of success in separating the protozoa from the cyst

membranes with adhering bacteria in the first of the two final washings in the centrifuge. The fluctuation in the angle of the $t_{50}$ curve on semilogarithmic paper with the axis of the abscissae is also probably due to an undetermined variation in technique. The steeper the line, the more powerful is the encystment-promoting action of an increase in the concentration of the organisms.

The encystment curves (Fig. 1) for different concentrations of *Colpoda* in drops of 1/40 cc. show that encystment is promoted by an increase in the concentration. In the lowest concentration (1,240 *Colpoda* per cubic centimeter) about 20 per cent of them can be expected to form resting cysts (Taylor and Strickland, 1939). Encystment is so slow that about two hours after the first cysts have appeared the other 80 per cent have begun to excyst, so that just after the point of 50 per cent encystment the curve begins to flatten. The three lower curves all show some excystment beginning at two hours after the first cysts were formed. The highest concentration (200,000 per cubic centimeter) should finally reach 100 per cent, and all should be resting cysts.

Figure 2 shows that the encystment time $t_{50}$ decreases proportionately with the increase in the logarithm of the concentration. This indicates an encystment-promoting conditioning of the environment by the organisms. The eight series in drops of 1/40 cc. vary considerably among themselves. The standard deviations and the standard errors for each group are high; since 5,000 *Colpoda* per drop is the maximum number that can be observed with sufficient speed and accuracy, the concentrations are comparatively low.

Figure 4 and Table 4 show that, when the points in Figure 4 are divided into six groups of concentrations, the mean $M$ of the standard deviations of the points in a group increases as the mean concentration of the group decreases. This shows that the encystment-promoting factor is more efficient in that it produces more uniform, as well as more potent, results in high concentrations of the protozoa.

If we take from the five curves in Figure 4 the values of $t_{50}$ at any one point, e.g., 100,000 *Colpoda* per cubic centimeter, we find

| | |
|---|---|
| 153.5 minutes for drops of | 1/40 cc. |
| 159.0 | 1/200 |
| 167.5 | 1/1,000 |
| 143.0 | 1/5,000 |
| 157.5 | 1/25,000 |

The mean is therefore 156 minutes $\pm 8$ minutes, or 156 minutes $\pm 5.1$ per cent. The standard deviation (8 minutes) is much smaller than the standard deviations of the five curves in this region (group III, Table 4), and these five standard deviations all overlap each other. There can be little doubt that under ideal conditions the five curves would coincide. The time required for 50 per cent of the *Colpoda* to encyst at any concentration of organisms is therefore independent of the volume of the drop within the range of concentrations and in the sizes of drop considered here. This indicates that the encystment-promoting factor is not identical with the factor inducing the change from cyst to resting cyst, whose potency varies with the volume of the drop.

If the curve illustrating the relationship between $t_{50}$ and the concentration continues unchanged, it should be theoretically possible to concentrate the protozoa so highly that when placed in food-free medium and with no unassimilated food in the body they

would immediately begin the secretion of a cyst membrane. In such a case the $t_{50}$ curve would approach the arbitrarily chosen zero line.

If the curve is extrapolated to a concentration of one *Colpoda* per cubic centimeter, the required $t_{50}$ should be about 250 minutes. One *Colpoda* in a liter of medium should require about 320 minutes. It seems, therefore, possible that the encystment-promoting factor, produced in the organism as an indirect result of an insufficiency of food, may condition the internal environment so as to induce encystment. If some of this promoting agent were lost by diffusion into the medium, the greater the concentration of organisms, the less would be this loss by diffusion.

At constant concentration of *Colpoda*—i.e., the number of organisms per unit volume of medium—the concentration of the factor in the medium due to diffusion from the protozoa would be independent of the volume of medium if the factor is not affected by the surface-volume ratio of the drop. This is in marked contrast with the properties of the factor determining the percentage of resting cysts, which is apparently greatly affected by the surface-volume ratio.

If a threshold concentration of the factor in the external environment were necessary to induce encystment, one would not expect the curves in Figure 1 to start at the same time; the beginning of encystment should rather be delayed, depending on the number of organisms per cubic centimeter, until the factor had reached the requisite concentration; the encystment curves would then all be parallel and similar.

## SUMMARY

This study of the time $t_{50}$ required for 50 per cent of various concentrations of *C. duodenaria* to form cysts in various volumes of food-free medium, reveals, for concentrations between 1,000 and 20,000,000 *Colpoda* per cubic centimeter, that (1) the time $t_{50}$ decreases proportionately to the increase in the logarithm of the concentration of organisms, (2) the time $t_{50}$ is independent of the volume of the drop containing the protozoa. The possibility of an encystment-promoting factor, indirectly induced by an insufficiency of food, is briefly discussed.

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